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Contents

Quantitative Histochemistry of the Nephron. VI. Hydroxyproline in the Human Glomerulus. SJOERD L. BONTING, HENDRINA DE BRUIN and VICTOR E. POLLAK 177
Serum Factors of Acquired Hemolytic Anemia in Leukemia and Lymphoma. JEROME I. BRODY and STUART C. FINCH 181
The Biosynthesis of Hemoglobin from Iron, Protoporphyrin and Globin. HERBERT C. SCHWARTZ, ROBERT GOUDSMIT, ROBERT L. HILL, GEORGE E. CARTWRIGHT and MAXWELL M. WINTROBE 188
Studies on the Response of the Transplanted Kidney and the Transplanted Adrenal Gland to Thoracic Inferior Vena Caval Constriction. CHARLES C. J. CARPENTER, JAMES O. DAVIS, JOHN E. HOLMAN, CARLOS R. AYERS and ROBERT C. BAHN 196
Studies on the Differences in Activity of Lung and Brain Thromboplastin. HARVEY J. WEISS, JAMES W. EICHELBERGER, JR. and WILLIAM H. CROSBY 205
The Effect of Induced Hyperammonemia on Renal Ammonia Metabolism. EDWARD E. OWEN, JAMES H. JOHNSON and MALCOLM P. TYOR 215
Lymphatic Flow in Human Subjects as Indicated by the Disappearance of I^{131}-Labeled Albumin from the Subcutaneous Tissue. W. HOLLANDER, P. REILLY and B. A. BURROWS 222
Ion Association. IV. Effect of Sodium Sulfate Infusion on Renal Clearance and Body Retention of Injected Radiostrontium in Dogs. MACKENZIE WALSER, JOHN W. PAYNE and ANN A. BROWDER 234
Studies on the Effects of Human Growth Hormone in Premature Infants. JACQUES R. DUCHARME and MELVIN M. GRUMBACH 243
Metabolism of 7-H^3-Epinephrine-d-Bitartrate in Normal Young Men. ELWOOD H. LABROSSE, JULIUS AXELROD, IRWIN J. KOPIN and SEYMOUR S. KETY 253
Studies of Anterior Pituitary Tissue *In Vitro*: Effects of Insulin and Experimental Diabetes Mellitus Upon Carbohydrate Metabolism. CHARLES J. GOODNER and NORBERT FREINKEL 261
Lung and Chest Wall Compliance of Apneic Paralyzed Infants. CHARLES C. RICHARDS and LEONARD BACHMAN 273
A Method for Determining Inert Gas ("N_2") Solubility in Urine. FRANCIS J. KLOCKE and HERMANN RAHN 279
The Arterial-Alveolar Inert Gas ("N_2") Difference in Normal and Emphysematous Subjects, as Indicated by the Analysis of Urine. FRANCIS J. KLOCKE and HERMANN RAHN 286
Observations on the Pyrogenic Response and its Application to the Bioassay of Endotoxin. WILLIS R. KEENE, HAROLD R. SILBERMAN and MAURICE LANDY 295
Inactivation of Endotoxin by a Humoral Component. VII. Enzymatic Degradation of Endotoxin by Blood Plasma. WILLIS R. KEENE, MAURICE LANDY and MURRAY J. SHEAR 302
Direct Renal Action of Some Digitalis Steroids. JAMES C. STRICKLER and RICHARD H. KESSLER 311
The Pulmonary Blood Volume in Man. DONALD S. DOCK, WILLIAM L. KRAUS, LOCKHART B. MCGUIRE, JOHN W. HYLAND, FLORENCE W. HAYNES and LEWIS DEXTER 317
A Method for the Determination of the Volume of Air in the Lungs: Measurements in Chronic Pulmonary Emphysema. G. EMMANUEL, W. A. BRISCOE and A. COURNAND 329
The Effects of Intravenous Infusions of Valine-5 Angiotensin II and Other Pressor Agents on Urinary Electrolytes and Corticosteroids, Including Aldosterone. P. BIRON, E. KOIW, W. NOWACZYNSKI, J. BROUILLET and J. GENEST 338
Pulmonary Function in Hyperthyroidism. MYRON STEIN, PHILIP KIMBEL and ROBERT L. JOHNSON, JR. 348
Studies of Glycogen Metabolism in Liver Glycogen Disease (von Gierke's Disease): Six Cases with Similar Metabolic Abnormalities and Responses to Glucagon. JOSEPH E. SOKAL, CHARLES U. LOWE, EDWARD J. SARCIONE, LUIS L. MOSOVICH and BERNARD H. DORAY 364
Interpretation of Changes in "Central" Blood Volume and Slope Volume During Exercise in Man. ROBERT J. MARSHALL and JOHN T. SHEPHERD 375
The Relation Between External Potassium Concentration and the Electrolyte Content of Isolated Rat Muscle in the Steady State. ARNOLD S. RELMAN, GEORGE W. GORHAM and NORMAN G. LEVINSKY 386
The Effect of Ethanol on Fatty Acid Metabolism; Stimulation of Hepatic Fatty Acid Synthesis *In Vitro*. CHARLES S. LIEBER and RUDI SCHMID 394
Studies on Pyrogenic Steroids. I. Separation, Identification, and Measurement of Unconjugated Dehydroepiandrosterone, Etiocholanolone, and Androsterone in Human Plasma. GEORGE L. COHN, PHILIP K. BONDY and CARMELA CASTIGLIONE 400
Body Fluid and Electrolyte Composition in Arterial Hypertension. I. Studies in Essential, Renal and Malignant Hypertension. W. HOLLANDER, A. V. CHOBANIAN and B. A. BURROWS 408
Body Fluid and Electrolyte Composition in Arterial Hypertension. II. Studies in Mineralocorticoid Hypertension. A. V. CHOBANIAN, B. A. BURROWS and W. HOLLANDER 416
The Incorporation of Radioactive Phosphorus into the Phospholipids of Human Leukemic Leukocytes and Platelets. BARRY G. FIRKIN and WILLIAM J. WILLIAMS 423
Studies of Bone of Patients with Hyperparathyroidism by Means of Phosphate Exchange Experiments *In Vitro* and Quantitative Microradiography. D. SMEENK 433
Announcement of Meetings 187

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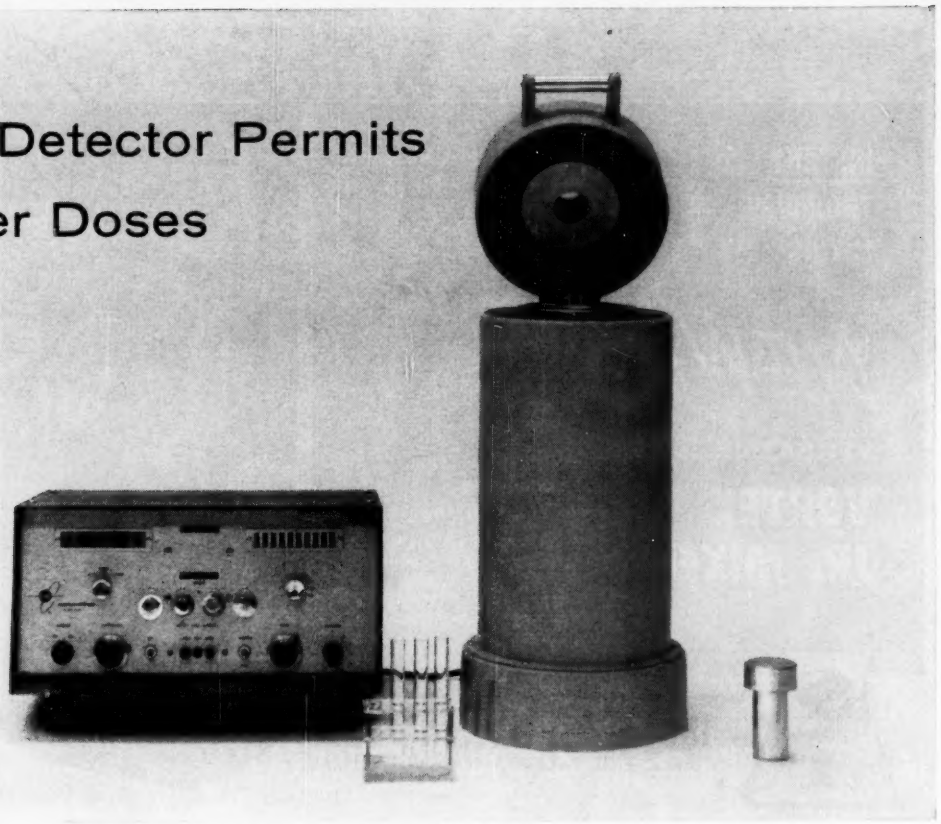

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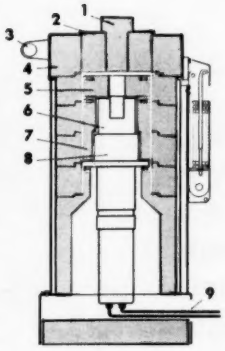

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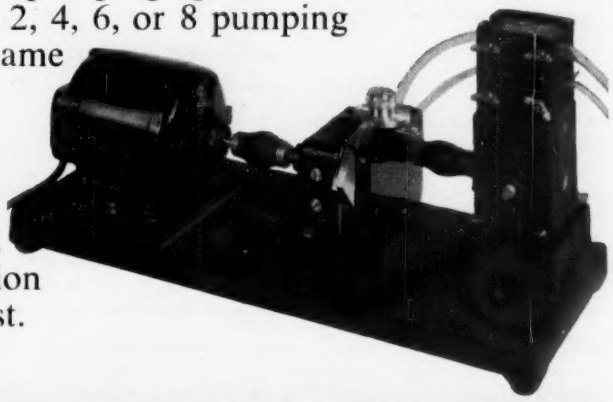

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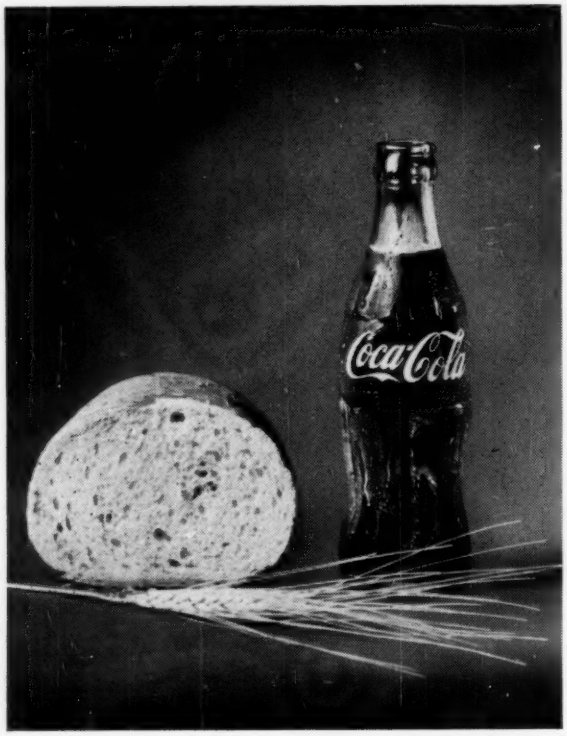

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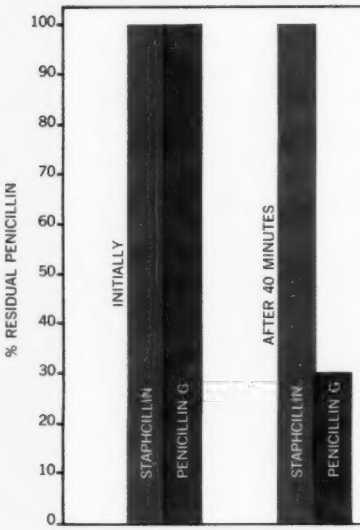

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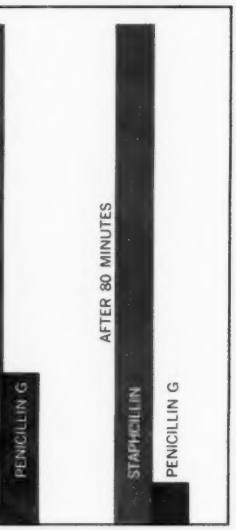

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REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

Janvier 1961

Vol. VI, No. 1

Editorial

La Chirurgie Endocrinienne du Cancer. P. JURET.

Original Articles

Formes Cliniques Psychosomatiques de l'Ulcus Gastro-Duodenal en Milieu Hospitalier. M. DE M'UZAN et S. BONFILS.

Détermination du Potassium Échangeable a Partir d'Échantillons Salivaires. C. NAGANT DE DEUXCHAISNES, R. BUSSET et R. A. COLLET.

Effet Protecteur de la Cystéamine et de la Cystamine sur les Thymocytes Irradies in Vitro. E. H. BETZ, G. BOOZ et P. LELIÈVRE.

L'Implantation de Matériel Radio-Actif Intrahypophysaire (198Au et 90y) dans les Cancers du Sein Métastatiques. A Propos de 75 Cas.

Original Notes

L'Inactivation Reversible de la Phosphatase Acide Prostatique: Action du pH, du Temps d'Incubation et du Serum Sanguin. H. TAGNON et A. STEENS-LIEVENS.

Dosage de l'Activite Lipasique Maxima du Suc Duodénal Recueilli par Tubage Apres Injection de Pancréozymine. J. GADRAT, P. FERRET, A. RIBET, R. BOURSE et BANIDE.

L'Influence de l'Obstruction Totale des Bronches sur la Mécanique Respiratoire. J.-M. VERSTRAETEN.

Sur le Mécanisme d'Action de la Guanathidine, Nouvel Agent Antihypertenseur. A. DRESSE.

Tests Directs d'Exploration de la Fonction Thrombodynamique des Plaquettes. G. MARCHAL, SAMAMA, LEROUX M. E., et R. J. PROSP.

L'Hyperlactacidémie, Cause Probable de l'Hypérurigémie dans la Glycogenose Hépatique. J. JEANDET et H. LESTRADET.

General Review

Les Enseignements de la Médecine Expérimentale dans les Problèmes de l'Hypertension. J.-F. CIER.

Technical Note

La Thymectomie Totale Chez le Souriceau Nouveau Ne. W. DISCHLER et G. RUDALI.

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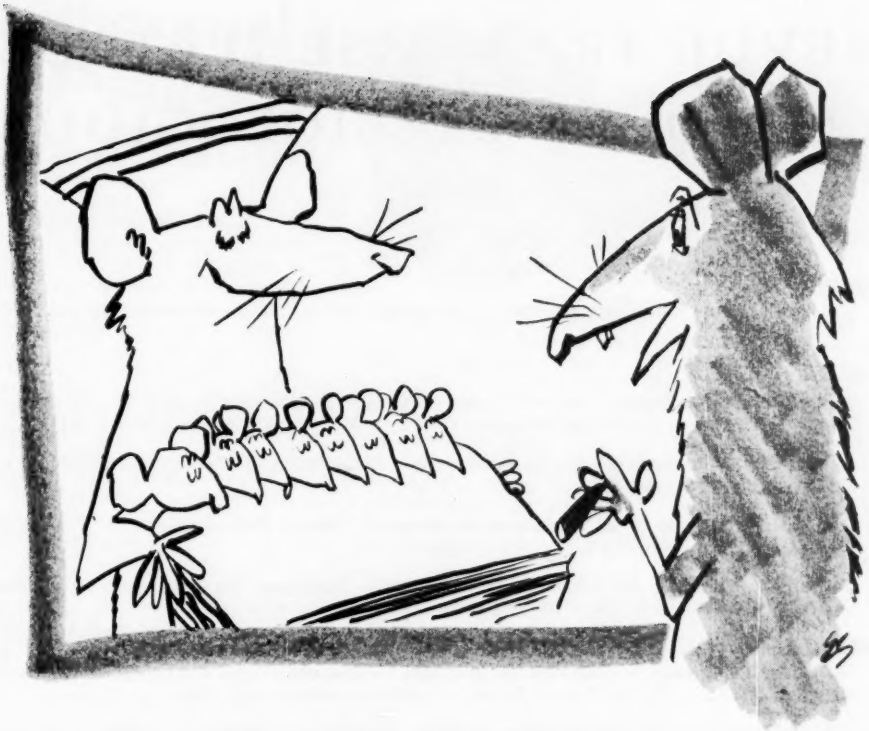

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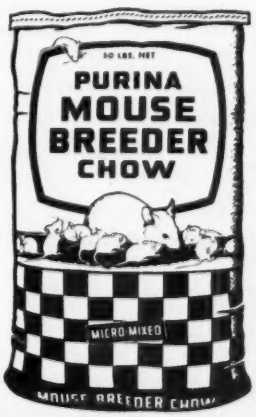

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February, 1961
Vol. VI, No. 2

Editorial

La Place Actuelle de l'Erythropoietine en Physiologie et en Clinique Humaine. Y. NAJEAN et R. ARDAILLOU.

Original Articles

La Permeabilite du Nephron pour les Ions Na^+, K^+ et Cl^-, Etudiee chez le Lapin a l'Aide d'Indicateurs Pendant l'Epreuve de Diurese Interrompue. Cl. AMIEL, M. GUINNEBAULT, C. BOUDIAK et F. MOREL.

Contribution a l'Etude du Granulome Experimental a Corps Etrangers (Technique Utilisant une Eponge de Polyvinyl). O. FLANDRE.

L'Ornithine Carbamyl (0 C) Transferase du Serum en Clinique Hepatologique. G. F. MORETTI, J. STAEFFEN, P. BALLAN, G. DEMANGE et J. ROUSSEAU.

Influence de Quelques Hormones Hypophysaires et Surrenaliennes sur la Colonisation d'Eponges en Ivalon Implantees chez le Rat. CH. M. LAPIÈRE et H. VAN CAUWENBERGE.

Original Notes

Technique Personnelle de Coloration des Globules Meterochromatiques des Leucocytes (Specialement des Mononuclees). Valeur de Cette Technique par la Determination du Sexe. G. SEMAN.

L'Identification de la Chromatine du Sexe dans les Chimeres Hematologiques Canines. G. MOLDOVANU.

Consommation de la Globuline Anti-Hemophilique et Pouvoir Thromboplastino-Accelerateur du Premier Produit Intermediaire (P.P.I.). G. MAYER, S. MAYER et R. WAITZ.

Modifications Avec l'Age du Rapport $\frac{\text{Galactosamine}}{\text{Glucosamine}}$ dans la Substance Fondamentale des Tendons et des Cartilages Articulaires. A. BERTOLIN.

Etude Electrophoretique de l'Hemoglobine du Sang Humain Vieilli et des Macerations de Taches de Sang Humain. M. MULLER, G. FONTAINE et H. MULLER.

Recherches sur les Auto-Anticorps au Cours de la Recto Colite Hemorragique et des Diverses Maladies Disgestives. Z. MARATKA et V. WAGNER.

General Review

Facteurs Biophysiques Conditionnant la Survie de la Souris Irradiee et Restauree par la Greffe de Cellules Hemopoietiques. G. DORIA.

Technical Note

Reaction de Waaler-Rose. G. L. DAGUET.

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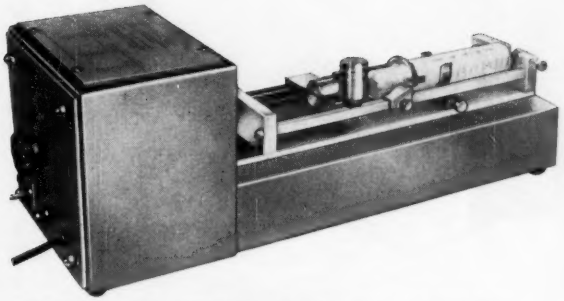

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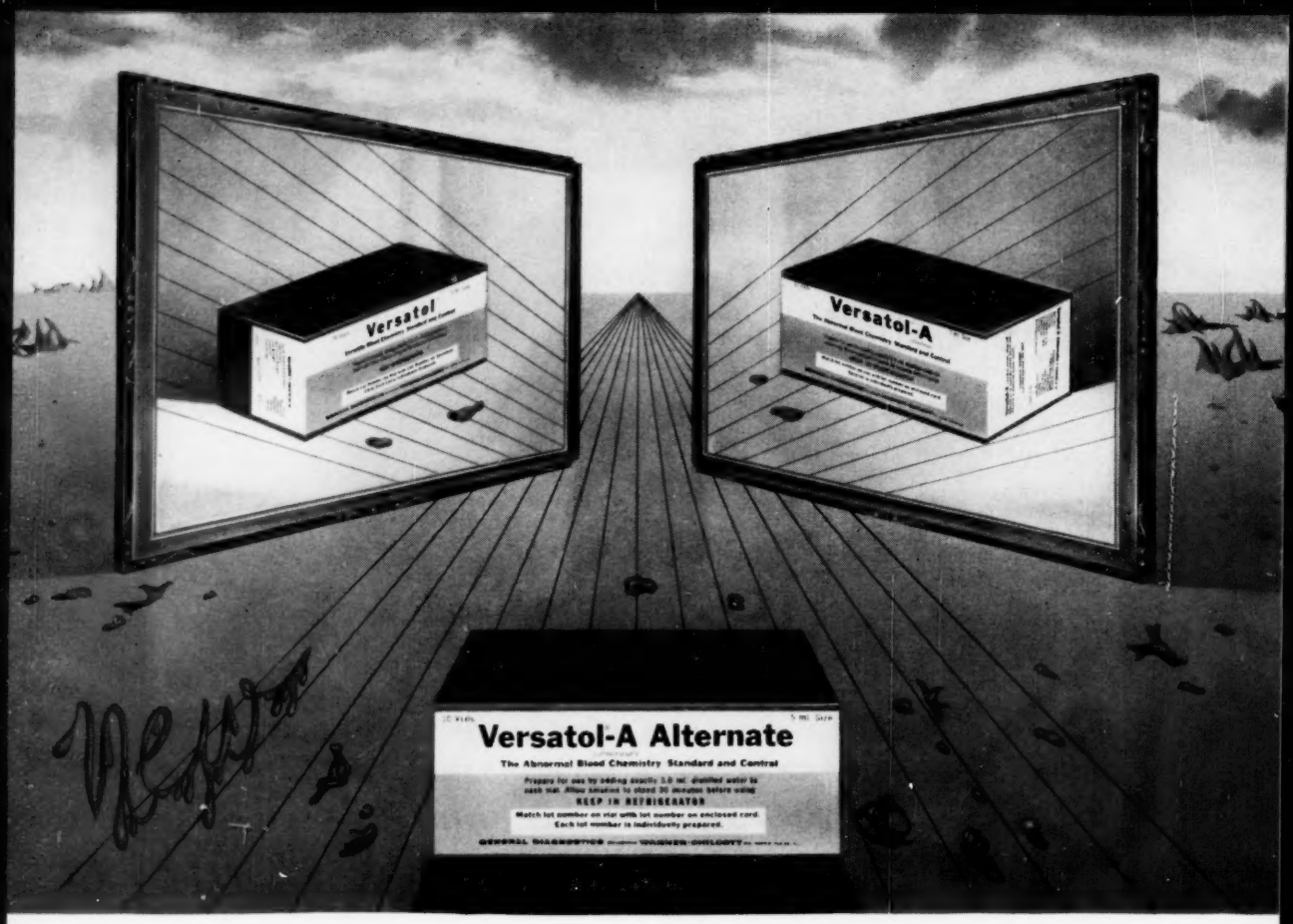

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QUANTITATIVE HISTOCHEMISTRY OF THE NEPHRON. VI. HYDROXYPROLINE IN THE HUMAN GLOMERULUS *

SJOERD L. BONTING,† HENDRINA DE BRUIN AND VICTOR E. POLLAK ‡

(From the Departments of Biological Chemistry and Medicine, University of Illinois College of Medicine, and Departments of Medicine, Presbyterian-St. Luke's Hospital, Cook County Hospital and Research and Education Hospitals, Chicago, Ill.)

(Submitted for publication June 23, 1960; accepted August 11, 1960)

Staining reactions characteristic of collagen and reticulin are found in pathologically altered glomeruli (1, 2), and fibrils with the periodicity of collagen fibrils have been observed occasionally in electron micrographs of severely damaged glomerular tufts. However, as far as we are aware, fibrils have not been found by light or electron microscopy in the normal glomerular tuft, except at the hilus; and, in particular, no fibrils with the periodicity of collagen fibrils have been seen in the normal glomerular capillary basement membrane (3–6).

Hydroxyproline estimations are widely used as a measure of collagen because no other known animal protein contains appreciable amounts of this amino acid (7, 8). Goodman, Greenspon and Krakower used sonic vibration to separate glomerular basement membrane from healthy canine glomeruli and found that the hydroxyproline content of their basement membrane preparations was 7 per cent (9). They suggested, therefore, that glomerular capillary basement membrane contained a considerable amount of collagen. As their results appeared to conflict with the morphological evidence, the hydroxyproline content of the human glomerulus was reinvestigated, using a microdissection and microassay technique in which glomerular tufts were dissected out intact and free of Bowman's capsules for hydroxyproline assay.

METHODS

Portions of human kidney were obtained postmortem and were frozen rapidly in liquid nitrogen, as described previously (10, 11). Sections were cut at 30 μ in a cryostat, were mounted on glass slides and were then lyophilized at $-20°$ C. From these lyophilized sections individual glomeruli were dissected out freehand under a dissecting microscope. The dissected glomerular tufts were free of Bowman's capsules. The glomeruli were pooled in groups of 12 to 20, weighed on a quartz fiber balance, and inserted into micro-test tubes for chemical analysis. In addition, portions of arcuate arteries were dissected out and analyzed separately.

Hydroxyproline was measured by a modification of Grunbaum and Glick's ultra-micro adaptation (12) of the method of Neumann and Logan (13). Twenty μl of 6 N HCl was added to the glomeruli contained in the micro-test tubes, which were then sealed and heated at 170° C for 1 hour. They were opened and the hydrolysate was evaporated to dryness over NaOH pellets in a vacuum desiccator. Ten μl of 5 mM $CuSO_4$ and 10 μl of a freshly prepared mixture containing 1.25 M NaOH, and 1 per cent H_2O_2 were added to each tube. The tubes were vibrated rapidly to stir the mixture, capped with parafilm, and heated at 75° C for 6 minutes. Next, 35 μl of a freshly prepared mixture containing 1.71 M H_2SO_4 and 1.14 per cent *p*-dimethylaminobenzaldehyde was added. The tubes were again vibrated rapidly to mix, capped and heated at 75° C for 15 minutes. The optical density of the resulting solution was read at 557 mμ. The color was found to be stable for at least 1 hour when the tubes were kept in an ice bath after color formation. The hydroxyproline concentration in tissue samples was expressed as a percentage of the dry weight.

Suitable blanks and standards were carried through the entire procedure including hydrolysis. The standard curves obtained were linear up to optical densities of 0.350. A commercial dried gelatin preparation (Knox) was found to contain 12.0 per cent hydroxyproline. When hydroxyproline was added to the gelatin a recovery of 105.6 per cent was obtained (equivalent, within the experimental error, to 100 per cent).

RESULTS

The hydroxyproline concentration in glomeruli from seven healthy kidneys was measured (Table I). The patients had died of causes other than renal disease, and had had no clinical evidence of renal damage. Their kidneys were normal macroscopically and the glomeruli were within normal

* Supported by a contract from the Surgeon General's Office, U. S. Army (Contract no. DA-49-007-MD-637), and by a grant from the U. S. Public Health Service, National Institutes of Health, Bethesda, Md. (H-3912).

† Present address: Section on Cell Biology, National Institute of Neurological Diseases and Blindness, Bethesda, Md.

‡ Established Investigator, American Heart Association, supported by the Illinois Heart Association.

TABLE I

Hydroxyproline concentration in the glomeruli and arcuate arteries of human kidney (expressed as a percentage of dry weight) *

	Hydroxyproline concentration
Normal human glomeruli	
Infant (1 day)	1.13 ± 0.073 [5]
Infant (5 months)	0.95 ± 0.101 [3]
Male (25 yrs)	1.11 ± 0.080 [10]
Female (26 yrs)	1.20 ± 0.25 [4]
Male (43 yrs)	1.08 ± 0.12 [4]
Male (65 yrs)	1.14 ± 0.094 [10]
Male (84 yrs)	1.15 ± 0.059 [9]
Average	1.109 ± 0.030
Abnormal human glomeruli	
Adult Fanconi syndrome (51 yrs)	1.57 ± 0.10 [6]
Nephrosclerosis (70 yrs)	1.65 ± 0.096 [3]
Diabetic nephropathy (50 yrs)	2.34 ± 0.19 [3]
Normal human arcuate arteries	
Male (25 yrs)	4.99 ± 0.65 [3]
Female (26 yrs)	3.88 ± 0.38 [4]
Male (65 yrs)	4.19 ± 0.96 [4]

* Results are expressed as the mean with the standard error and, in brackets, the number of determinations. Each determination was made on a pooled sample of approximately 20 glomeruli.

limits microscopically. The hydroxyproline concentration was found to be 1.109 ± 0.030 (standard error of the mean) per cent of the dry weight. This is equivalent to 8.9 per cent of collagen on the basis of a hydroxyproline concentration in collagen of 12.5 per cent (8). For comparison, the hydroxyproline concentration in renal arcuate arteries was measured and was found to be from 3.88 to 4.99 per cent of dry weight (Table I). This is equivalent to 31.0 to 39.9 per cent of collagen.

The hydroxyproline concentration in the glomeruli of three abnormal kidneys was also measured (Table I), and was found to be significantly increased in all. The highest hydroxyproline concentration was found in the glomeruli of a patient with severe diffuse and nodular diabetic glomerulosclerosis. The hydroxyproline concentration was also increased in the glomeruli of a patient with moderately severe nephrosclerosis and diffuse thickening of the glomerular basement membrane, and in the glomeruli from a patient with multiple myeloma and the adult Fanconi syndrome.

DISCUSSION

The results indicate that there are significant amounts of hydroxyproline in the normal glomerulus. The hydroxyproline concentration in the glomeruli studied was remarkably constant from one individual to another, despite the wide range of the ages (from 1 day to 84 years) of the subjects studied; there was no increase of hydroxyproline concentration with increasing age. The glomerular basement membrane of man increases in thickness from birth (approximately 950 Å) to three years of age, when the adult thickness of 3,000 Å is attained (14). If the hydroxyproline is located in the glomerular basement membrane, the hydroxyproline content of the whole glomerulus might be expected to increase from birth to three years of age. However, it must be remembered that all elements of the glomeruli increase in weight during growth. Elsewhere we have shown that the average dry weight of 130 healthy glomeruli dissected out from 16 μ sections was 42.1 mμg in the adult (15); that of 106 healthy glomeruli similarly dissected out from 16 μ sections of kidney in infants (1 day to 5 months old) was 24.3 mμg. This resolves partially the apparent contradiction between our chemical findings and the electron microscopic observations. Another factor may be a difference in shrinkage for material from infants and adults during osmium tetroxide fixation, alcohol dehydration, and embedding for electron microscopy.

The proportion of hydroxyproline was increased in certain pathological conditions of the glomerulus characterized histologically by thickening of the capillary basement membrane and vascular sclerosis. This finding was consistent with histological studies of the abnormal glomeruli, using the Mallory azan technique, in which an increased amount of blue staining material, typical of collagen, was found.

The only other reported chemical analysis of hydroxyproline in the glomerulus is that of Goodman and associates (9). These authors obtained glomeruli in bulk by screening and centrifugation of the renal cortex. From the isolated glomeruli, glomerular basement membrane was obtained by sonic vibration; it was analyzed for hydroxyproline (6.9 to 7.5 per cent), collagen (79.6 to 83.6 per cent), and total nitrogen (11.4 to 12.3 per cent). Thus, they recorded a hydroxyproline content in isolated glomerular basement membrane approximately 6.5 times higher than that found in the present investigation in whole glomeruli (without capsule).

The presence of endothelial and epithelial cells in glomeruli prepared according to our method and their absence from the preparations analyzed by Goodman and co-workers, partially explains the difference. However, the magnitude of the difference suggests that other factors may be important, such as solubilization of noncollagen protein during the isolation procedure used by these authors.

To elucidate this difference, whole glomeruli from a healthy male, aged 25 years (Table I), were analyzed for hydroxyproline: *1*) after isolation by screening and centrifugation by the technique of Goodman and associates (9), followed by freeze-drying (3.23 ± 0.21 per cent); and *2*) after dissection from frozen-dried sections (1.11 ± 0.08 per cent). The threefold difference found in the two methods of preparation of the tissue for analysis is not likely to be due to the inclusion or omission of Bowman's capsules, since it has been shown that the hydroxyproline concentration in glomeruli is not significantly different, whether or not the glomeruli are dissected free of Bowman's capsules before analysis (9). It is explained most readily by solubilization of noncollagen protein during the wet isolation procedure. This view is supported by the ratio of collagen to total nitrogen in the experiments of Goodman and co-workers, which suggests that little or no noncollagen protein was present in the glomerular basement membrane preparations analyzed by them.

The absence of electron microscopic evidence of a fibrillar structure in the glomerular basement membrane might suggest that there is no collagen in this structure, and the demonstration of a hydroxyproline content of 1.109 per cent might appear to be in conflict with the electron microscopic observations. This apparent contradiction can be reconciled by assuming that the basement membrane contains a nonfibrillar, hydroxyproline-rich protein. This view is consistent with the solubility and enzymatic digestibility of Greenspon, Bollinger and Krakower's basement membrane preparation (16).

Most electron microscopic studies of collagen have been made on material extracted from skin or tendon. Jackson has suggested that intracellular collagen is not fibrous and that it is excreted in this form into the extracellular spaces from which it can be extracted as a neutral salt-soluble collagen (17). Both he and Gross (18) have shown that neutral salt-soluble collagen has the consistency of a gel *in vitro*, which at times may polymerize or precipitate spontaneously into typical collagen fibrils. Moreover, neutral salt-soluble collagen appears to be a precursor *in vivo* of the form with 640 Å banded fibrils (18). Thus, the electron microscopic observations and the biochemical findings reported here could be reconciled by assuming that the normal glomerular basement membrane contains a nonfibrillar form of collagen similar to the neutral salt-extractable, soluble collagen of Jackson and Gross (17,18), and that the the fibrillar form is laid down only under pathological conditions.

SUMMARY

1. The hydroxyproline concentration in glomerular tufts from nine healthy kidneys was measured, and was found to be 1.109 per cent of the dry weight. Its concentration was not related to the age of the subjects. In renal arcuate arteries the hydroxyproline concentration was 3.88 to 4.88 per cent of dry weight. In abnormal glomeruli from three subjects the hydroxyproline concentration was significantly higher than in normal glomeruli, the highest concentration being found in a case of diabetic nephropathy.

2. These findings indicate that collagen is present in the glomerular basement membranes. The absence of fibrils, which has been demonstrated by the electron microscope, suggests that the collagen is present in a form which is a precursor of that with 640 Å banded fibrils, possibly neutral salt-extractable collagen.

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CORRECTION

On page 1597 of the paper entitled "Serum factors in lupus erythematosus and other diseases reacting with cell nuclei and nucleoprotein extracts: Electrophoretic, ultracentrifugal and chromatographic studies" by Howard C. Goodman, John L. Fahey and Richard A. Malmgren (J. clin. Invest. 1960, **39**, 1595), lines 15–18 should read: "Anti-calf thymus nucleoprotein extract activity was present in 85 per cent of SLE sera, 10 per cent of the RA sera and 25 per cent of the scleroderma sera." An error in typesetting omitted a line which confused the meaning of this sentence. Also, on page 1600 of the same article, the first sentence at the top of column 2 should read: "The proteins eluted first are seen to sediment less rapidly ($s^{\circ}_{20,w} \cong 6.6S$) than those eluted in the 60 to 80 per cent effluent region ($s^{\circ}_{20,w} \cong 18S$)."

SERUM FACTORS OF ACQUIRED HEMOLYTIC ANEMIA IN LEUKEMIA AND LYMPHOMA *

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The purpose of this study was to determine whether the abnormal serum factors of acquired hemolytic anemia in patients with leukemia and lymphoma could be characterized as antibody by the immune adherence technique. The pathogenesis of this disorder has not been completely explained and it was felt that the study of specific immunologic reactions might help to determine whether these serum factors are erythrocyte antibody. The technique of immune adherence (1, 2) was used not only because its flexibility and sensitivity make it most suitable for the study of antigen-antibody reactions but particularly because, insofar as is at present known, immune adherence will not occur unless antigen and specific complement-fixing antibody are present. The basic premise of this reaction states that in the presence of antigen, specific antibody, complement, and indicator (either primate red cells or nonprimate platelets), the antigen will adhere to the indicator particles. Immune adherence may be determined either macroscopically by red cell floccule formation or microscopically by dark-field illumination.

METHODS AND MATERIALS

A. Patient group

Serum samples taken from a total of 7 patients with acquired hemolytic anemia, ranging in age from 45 to 75 years, were studied. Two of the patients had lymphocytic lymphoma, 1 had Hodgkin's disease, 2 had chronic lymphocytic leukemia, 1 had acute monocytic leukemia and 1 had multiple myeloma. One patient with lymphoma had been treated with X-ray, nitrogen mustard, adrenocortical steroids and blood transfusions. Blood transfusions also were given to the patient with multiple myeloma. None of the other patients received either tumor therapy or blood transfusions.

B. Tests for sensitization

Direct and indirect antiglobulin tests were performed as described by Coombs, Mourant and Race (3) and Jandl and Castle (4). Both the antihuman globulin and patient serums were used at full strength only and neither direct nor indirect Coombs titers were determined.

C. Preparation of reactants

1. Indicator. Guinea pig platelets were used as indicator particles. They were collected and handled as described by Nelson and Nelson (5). Eight ml of Alsever's solution, previously chilled in ice, was drawn into a 20 ml syringe which had been kept in a freezer for 30 minutes. Eight ml of cardiac blood from a 500 g guinea pig was aspirated directly into the ice-cold solution. The blood then was transferred to a 30 ml, iced, Pyrex test tube. All centrifugation was done at 0° C. The blood-Alsever's solution was centrifuged at 80 G for 30 minutes. The supernatant was removed carefully with a capillary pipet and transferred to a 40 ml lipped, round-bottom tube and centrifuged at 900 G for 20 minutes. The supernatant again was removed and discarded. The platelet button was resuspended by gentle agitation and the dropwise addition of 5 ml of cold Alsever's solution. The spinning and washing were repeated twice. The platelet button finally was resuspended in 2 ml gelatin veronal buffer (GVB) and centrifuged at 35 G for 5 minutes in order to remove any platelet clumps. This method resulted in minimal platelet clumping and gave relatively homogeneous platelet suspensions. The supernatant fluid, containing only platelets, was standardized to 50 per cent transmission on a Beckman DU spectrophotometer at a wave length of 400 mμ.

2. Antigen. Normal, type O, Rh negative erythrocytes, both unsensitized and sensitized with patient serum, and Coombs positive patient erythrocytes were used as antigen. The blood was collected directly into Alsever's solution (2 ml Alsever's to 1 ml whole blood) and was either used the same day or stored at 4° C for a maximum of 48 hours. In the latter instance, 250 U of aqueous penicillin G per ml blood-Alsever's solution was added to inhibit bacterial growth. The red cells were washed three times with GVB and made up to a 2 per cent solution in GVB. In this assay they were used

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TABLE I

Checkerboard titration in immune adherence

Antigen*		Antibody† dilution				
Concentration	Dilution	1:100	1:200	1:400	1:800	1:1,600
%						
2	Undiluted	+2	+3	+4	+3	+2
2	1:2	+1	+1	+2	+3	+1
2	1:4	0	0	+1	+1	0

* Human, type A erythrocytes.
† Homologous, type A antiserum.

undiluted at 2 per cent, and at dilutions of 1:2 and 1:4 of the 2 per cent suspension with GVB.

3. Complement. Normal, human, type AB, Rh negative serum was used as the complement source. It was kept at −70° C in 1-ml aliquot portions in screw-top vials. After quick thawing it was used in a constant dilution of 1:40 with GVB.

4. Antibody. In order to determine the suitability of the immune adherence (IA) technique for the detection of erythrocyte antibody, heterologous typing serums such as anti-M and anti-N were used as antibody source in dilutions of 1:100 and 1:1,600. Human, type-specific anti-A and anti-B serums of the natural and immune type and both agglutinating and incomplete (nonagglutinating) anti-Rh_0 (D) serums were used as homologous antibody in the same dilutions. An initial dilution of 1:100 was used because, except for the incomplete anti-Rh_0 (D) serum, in dilutions below this concentration, the red cells were agglutinated spontaneously by the antiserums. In an analogous manner, patient serums and a broad-spectrum antihuman globulin [1] were used as antibody source in dilutions of 1:10 to 1:160. This dilution range was employed because it afforded optimal reactivity in the assay. Conventional agglutination titers were not performed on the individual antiserums.

D. Performance of the assay

All reactants were kept at 0° C in crushed ice prior to their combination. Screw-top vials with a 2 ml capacity were used in the test. Regardless of its source, 0.25 ml of antibody in the dilutions noted previously was added slowly to 0.25 ml-aliquots of each red cell dilution (antigen) so that a checkerboard pattern titration resulted. This type of titration was constructed because optimal reactivity in IA is dependent in large part upon the relative concentrations of antigen and antibody. The vials were rotated at 6 rpm in a 37° C incubator for 10 minutes. Upon removal, 0.5 ml of chilled complement (1:40 dilution) was added to the antigen-antibody mixture in each vial. The 10 minute incubation, with slow rotation, was repeated and 0.1 ml of the indicator platelet suspension then was added to each vial. The vials were rotated at 37° C for 40 minutes and removed from the incubator. Wet coverslip preparations, made by placing one drop of the incubation mixture on a glass slide, were examined microscopically under dark-field illumination. IA of the red cells to the platelets indicated the presence of erythrocyte antibody. The reaction was read as negative when no red cells were adherent to the platelets, as a trace when less than 10 per cent of the red cells adhered to the

[1] Supplied by the Ortho Pharmaceutical Corporation, Raritan, N. J.

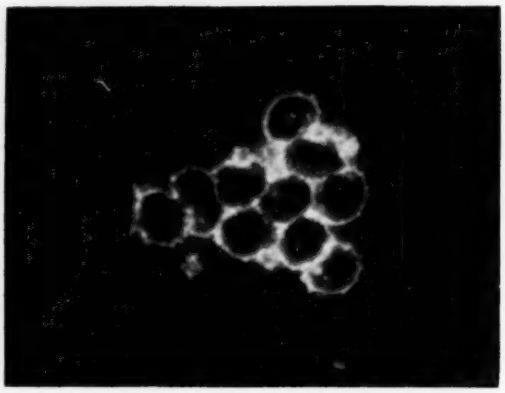

FIG. 1. A POSITIVE IMMUNE ADHERENCE REACTION UNDER DARK-FIELD MICROSCOPY. The erythrocytes are adherent to the guinea pig platelets which are seen as luminescent particles between the red cells (×450).

TABLE II

Type-specific erythrocyte immune adherence reactions

Antigen	Antibody	Antibody dilution				
		1:100	1:200	1:400	1:800	1:1,600
M erythrocytes	Heterologous anti-M	+2	+2	+4	+3	+2
N erythrocytes	Heterologous anti-N	+2	+4	+3	+2	+2
A erythrocytes	Homologous anti-A	+2	+3	+4	+3	+2
B erythrocytes	Homologous anti-B	+2	+3	+4	+3	+2
Rh_0 (D) erythrocytes	Homologous anti-Rh_0 (D)					
	Agglutinating	+2	+2	+4	+2	+2
	Incomplete (nonagglutinating)	+1	+1	+2	+1	0

platlets, + 1 when there was IA of 10 to 25 per cent of the red cells, + 2 with IA of 25 to 50 per cent of the red cells, + 3 with IA of 50 to 75 per cent of the red cells, and + 4 with IA of more than 75 per cent of the erythrocytes. An example of this type of titration is illustrated in Table I.

RESULTS

A. Tests for erythrocyte sensitization. All patients except the one with Hodgkin's disease had positive direct and indirect antiglobulin reactions. There was good agreement between the antihuman globulin (3) and the polyvinylpyrrolidone (4) tests in these patients.

B. IA antibody assays. IA occurred when heterologous anti-M and anti-N and homologous anti-A, anti-B and both types of anti-Rh_0 (D) serums were reacted with erythrocytes of the appropriate antigenic structure (Figure 1). Except for anti-N and incomplete anti-Rh_0 (D), the reactions were + 4 at dilutions of 1 : 400 and + 2 at dilutions of 1 : 1,600 in both the heterologous and homologous titrations. Dilutions of anti-N sera revealed a + 4 reaction at 1 : 200 and + 2 at 1 : 800. The incomplete (nonagglutinating) anti-Rh_0 (D) serum from a sensitized female gave a + 2 IA reaction in serum dilutions of up to 1 : 400. Control tubes lacking either complement or antibody were negative for IA. These results are summarized in Table II. IA did not occur when patients' serums were reacted with their own erythrocytes or when untransfused patient serum was reacted with normal erythrocytes. In the patient with Hodgkin's disease, who had negative direct and indirect Coombs reactions, IA did not occur in any of the antigen-antibody combinations employed. However, when the serums of the two patients who had been repeatedly transfused were reacted with normal red cells, IA (+ 2) did occur in dilutions of 1 : 160. IA was also noted when antihuman globulin was reacted with serum-sensitized normal and Coombs positive patient erythrocytes. The patterns in these assays revealed a + 4 reaction at a 1 : 40 dilution and a + 1 at a 1 : 160 dilution. Control tubes lacking antibody or complement were negative. These results are summarized in Table III.

DISCUSSION

The occurrence of IA when type-specific heterologous and homologous antiserums were reacted with appropriate erythrocytes indicates that this method is a sensitive indicator for the detection of natural and immune erythrocyte antibody. The increased sensitivity of IA as compared to conventional agglutination techniques permitted demonstration of antibody titers up to a dilution of at least 1 : 1,600. Maximum anti-N titers in a dilution of only 1 :800 may depend on the diminished antigenicity of the N substance when compared to other blood-group factors. The most significant observation of this study was the absence of IA when serum from patients with acquired hemolytic anemia was reacted with either their own or normal erythrocytes. This indicated that a specific antigen-antibody reaction did not occur and that erythrocyte antibody was not present in these patients as measured by the IA technique, despite positive direct and indirect Coombs antiglobulin reactions. The test originally described by Coombs, Mourant and Race (6) was a new one for the detection of "incomplete" Rh agglutinins. Subsequently, other laboratory methods have been devised to demonstrate possible erythrocyte sen-

TABLE III

Erythrocyte immune adherence in acquired hemolytic anemia (AHA)

Antigen	Antibody	Antibody dilution				
		1:10	1:20	1:40	1:80	1:160
AHA erythrocytes	AHA serum	0	0	0	0	0
Normal erythrocytes	Nontransfused AHA serum	0	0	0	0	0
Normal erythrocytes	Transfused AHA serum	0	0	+1	+2	+2
AHA serum-sensitized normal erythrocytes	Antihuman globulin	+1	+3	+4	+3	+1
Coombs positive patient erythrocytes	Antihuman globulin	+2	+3	+4	+2	+1

sitization or coating. These modifications have certain disadvantages because they may depend on alteration of the red cell surface or on high molecular substances used as suspending media. For example, a positive direct antiglobulin test may be obtained when the red cells are incubated with phenylhydrazine (7), upon exposure to heavy metals (8, 9) or when the red cell surface is altered by enzymes such as trypsin (10). Agglutination of erythrocytes in albumin-type media may be unrelated to erythrocyte sensitization (11). Many normal human serums have an auto-agglutinating property which has never been adequately explained (12). Silicates in colloidal solution may cause red cells to absorb serum protein *in vitro* and false positive antiglobulin reactions may occur (13). A positive direct antiglobulin test, therefore, does not necessarily imply that the reaction is immunologic in nature. IA occurred when the serum of patients who had received multiple transfusions was reacted with normal washed erythrocytes. It is probable that IA detected incomplete antibodies which had developed in response to weak erythrocyte antigens. These antibodies could not be detected during usual blood typing procedures and were not sufficiently reactive to cause a clinical transfusion incompatibility. Although isosensitization can occur following multiple transfusions, this is not the mechanism of the acquired hemolytic anemia secondary to neoplastic disease.

The presence of IA when antihuman globulin was reacted with experimentally or naturally sensitized erythrocytes was of considerable interest. Positive reactions were noted in antibody dilutions up to 1 : 160. In this type of IA reaction the globulin coating the red cell surface acts as antigen with the heterologous antibody in the Coombs serum. The major portion of the coating material which can be eluted from sensitized red cells appears to migrate electrophoretically with the gamma globulin fraction of the plasma proteins although alpha and beta globulins also may be found in the same eluate (14–16). After ultracentrifugation, the gamma globulins are present both in the 7S fraction and in the 19S fraction (17) suggesting that these materials constitute a somewhat heterogeneous group. Most antihuman globulin serums now available include antibodies against the gamma and non-gamma globulin serum fractions (18), providing additional evidence that this IA reaction was antigen-antibody in nature.

There was an awareness during this study that the absence of IA when patient serums were reacted with their own or normal erythrocytes might perhaps be due to a noncomplement-fixing antibody not active in the IA system. Consequently, because it is incomplete, because it is demonstrable by antihuman globulin, and because it was thought not to fix complement, nonagglutinating anti-D was employed as a prototype antibody in an attempt to reconcile this problem.

The observation that IA did occur when incomplete (nonagglutinating) anti-D was reacted with appropriate erythrocytes was important for two reasons. First, it demonstrated, by IA at least, that this antibody does fix complement. In this regard it should be pointed out that failure to demonstrate complement fixation with prior assays may have been due to the relatively insensitive methods utilized for this determination. Although the IA technique has not been widely employed in clinical investigation, it is, nevertheless, a well known, well accepted, generalized immunologic phenomenon which will not occur in the absence of specific complement-fixing antibody (19). This method is more than ten times as sensitive as standard immunologic procedures now employed (20) and has detected antibodies against treponemes (1), bacteria (2), fungi (21), starch (21) and viruses (22). In addition, IA with incomplete anti-D, which resembles in certain limited respects the serum factors of this type of acquired hemolytic anemia, supports the assumption that antibody directed against erythrocytes was not present in the serum of these patients. This does not, of course, completely deny the existence of an antibody which is not detectable by this particular immunologic method.

The concept of auto-antibody formation (23, 24) has been invoked repeatedly to explain the pathogenesis of the serum factors responsible for acquired hemolytic anemia. This hypothesis is based on the following observations. The materials present in the serum may be gamma globulins (25) which behave *in vitro* to cause either direct or indirect agglutination of red cells with antihuman globulin. Also, these factors may be eluted from coated red cells in a fashion similar to that for antibody elution (26). It is believed that this

serum auto-antibody becomes attached to red cells *in vivo* resulting in their premature sequestration and removal from the circulation (27, 28). This erythrocyte auto-antibody hypothesis has been strengthened by analogy to the experimental demonstration of auto-antibody formation to thyroid gland (29), lens (30), neurologic tissue (31) and sperm (32).

Although there are many similarities between acquired hemolytic anemia and auto-immune disorders, the analogy is not complete and there are a number of reasons which suggest that these abnormal serum factors are not erythrocyte auto-antibody. In order to fulfill the strict criteria for antibody a factor must be shown to be an immunologic response of the organism to a specific antigen. Furthermore, this serum antibody must be shown to react with the original antigen (33). Experimental attempts to fulfill these requirements have either failed (34) or have been only partially successful (35–37). The ability of eluates from Coombs positive erythrocytes to produce active antiglobulin serum (38) and also to fully coat and sensitize all human erythrocytes (39) is recognized, but this does not necessarily establish an antigenic relationship with red cells. In addition, sensitization of normal red cells by concentrated eluates from a patient with Coombs negative acquired hemolytic anemia did not cause significant alteration of the *in vivo* survival of the red cells. On the other hand, when type D red cells were sensitized by the concentrated eluate from anti-D coated erythrocytes, such cells disappeared rapidly from the circulation and were sequestered in the recipient's spleen (40). The auto-antibody concept of disease has been extended clinically to include idiopathic thrombocytopenic purpura (ITP) and some leukopenic states. The association of acquired hemolytic anemia with ITP is not uncommon (41) and occasionally it has been reported with immunoleukopenia or immunopancytopenia (42). Although concomitant auto-antibody production to each of the formed elements of the blood may be possible, it seems highly improbable that this type of immune response would occur.

The failure to characterize the serum factors capable of red cell sensitization as erythrocyte antibody by the IA technique in these patients suggests that they are not the result of antigenic stimulation by red cells. However, an alternate hypothesis may be offered to explain this syndrome. In patients with lymphoproliferative and related disorders there may be an as yet undertermined, abnormal stimulus to or distortion of the antibody-producing mechanisms of the body. This may allow the formation of certain globulins or factors, which may be antibody in nature, but whose antigenic relationships remain unrecognized. It has been demonstrated experimentally in animals that specific immunization is accompanied not only by specific antibody formation but also by the appearance of gamma globulins nonreactive with the original challenge (43–45). A clinical counterpart of this experiment may perhaps be seen in some patients with primary atypical pneumonia and infectious mononucleosis who reveal certain serologic abnormalities such as rising heterophile titers and biologically false positive tests for syphilis and who also develop transient acquired hemolytic anemia (46–49). Finally, to complicate this multi-faceted problem still further, the *de novo* synthesis of humoral antibody in patients with neoplastic disease is often impaired (50, 51), and yet it is this type of patient who not infrequently develops acquired hemolytic anemia.

The observations noted in this study do not disprove the auto-antibody hypothesis of acquired hemolytic anemia nor do they necessarily relate either to idiopathic acquired hemolytic anemia or acquired hemolytic anemia associated with other disease states, since these disorders have not been investigated. They do, however, provide additional support for the concept that these serum factors in lymphoma and leukemia are not erythrocyte auto-antibody (52, 53). It is suggested that these serum factors, having an affinity for the erythrocyte surface, may represent another manifestation of the dysproteinemic state.

SUMMARY

1. The purpose of this study was to determine whether the abnormal serum factors of acquired hemolytic anemia in patients with leukemia and lymphoma could be characterized as erythrocyte antibody by the immune adherence technique.

2. The serum factors capable of red cell sensitization in these patients did not react as erythrocyte antibody in immune adherence.

3. It is suggested that these factors, which have an affinity for the erythrocyte surface, may represent another manifestation of the dysproteinemic state.

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ANNOUNCEMENT OF MEETINGS

The Fifty-Third Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Sunday afternoon and evening, April 30, 1961, in the Chalfonte-Haddon Hall (jointly with the AFCR); and on Monday, May 1, at 9:00 a.m., at the Casino Theater on the Steel Pier.

The Eighteenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., on Sunday, April 30, 1961, at 9:00 a.m., at the Casino Theater on the Steel Pier. On Sunday afternoon and evening, April 30, 1961, a joint sectional meeting with THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in rooms in the Chalfonte-Haddon Hall.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold its Seventy-Fourth Annual Meeting in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 2, 1961, at 9:30 a.m., and in the Carolina Room, Chalfonte-Haddon Hall, on Wednesday, May 3, 1961, at 9:30 a.m.

THE BIOSYNTHESIS OF HEMOGLOBIN FROM IRON, PROTOPORPHYRIN AND GLOBIN * †

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The formation of heme in a system containing protoporphyrin, iron and a soluble preparation from avian erythrocytes has been studied by several investigators (3–6). The observations that the biosynthetic reaction is inactivated by heating at 56° C for 30 minutes, has a pH of optimal activity and stability near neutrality with a lower rate on each side of this optimum, and that the rate of heme synthesis is proportional to enzyme concentration, suggest, although admittedly do not prove, that the reaction is enzyme-dependent (4–6). The enzyme has been tentatively named "heme synthetase" (2, 5). However, since more than one enzyme may be involved in this reaction, the name "heme synthetase system" would be more appropriate until it can be shown that a single enzyme is involved.

The activity of the heme synthetase system is increased by reduced glutathione, cysteine, or other reducing agents (4–7). Inhibition with *p*-chloromercuriphenylsulfonate and with iodoacetamide suggests that active sulfhydryl groups are required for enzyme activity (5).

Purification of the heme synthetase system and augmentation of heme synthesis by globin are described in the present studies. Although several investigators have studied the synthesis of heme and globin in whole cell systems (8, 9), the effect of globin on the enzymic synthesis of heme in a purified system has not been demonstrated previously.

METHODS

Assay procedure

Reaction mixtures contained 6 ml of enzyme preparation, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, and 1,000 μmoles Tris [tris (hydroxymethyl) aminomethane] buffer, pH 7.8, in a total volume of 10 ml. The specific activity of the Fe^{59} ranged from 1 to 3 mc per mg of iron. The reaction mixtures were incubated with shaking at 37° C for 30 minutes. The reaction was then stopped by adding 25 ml of a "carrier" hemoglobin solution which contained 1×10^{-3} M potassium cyanide. The heme was crystallized twice and the radioactivity was determined (3, 5). Heme synthesis was expressed as the per cent incorporation of added radioiron into heme (per cent uptake of Fe^{59}) or as the total radioactivity in the heme (cpm). A single pool of chicken blood was used for a given experiment. In this way variations in iron uptake due to differences in the size of the iron pool from one sample of blood to another were eliminated.

Source of materials

Washed chicken erythrocytes, prepared as described previously (3, 5), were used as a source of enzyme. Purified albumin and γ-globulin were obtained from human plasma by preparative curtain electrophoresis (10). The transferrin used was Cohn fraction IV-7 (11). Globin was prepared by acid acetone precipitation (12). Saponin (Eastman Kodak Company) and Tween-20 (Atlas Powder Company) were obtained from commercial sources.

Preparative procedures

Starch block electrophoresis was carried out as described by Kunkel, Cepellini, Müller-Eberhard and Wolf (13). Column chromatography was performed as described by Allen, Schroeder and Balog (14). Except where stated, all of the preparative procedures were performed at 4° C.

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Analytical procedures

Hemoglobin concentrations were determined by the cyanmethemoglobin method in a Beckman model DU spectrophotometer (15). Nitrogen analyses were performed by a micro-Kjeldahl method (16).

Preparation of enzyme solutions

Step 1: preparation of hemolysate. Forty ml of washed, packed erythrocytes was hemolyzed (17) with 60 ml of 0.15 M KCl containing sufficient saponin to give a final saponin concentration of 0.03 per cent. This concentration of saponin was found to give maximal hemolysis with minimal enzyme inactivation. The hemolysate (fraction I) was incubated at 5° C for 30 minutes with intermittent mixing. One hundred ml of cold 0.15 M KCl was added and the hemolysate was centrifuged at 1,300 G for 60 minutes. The supernatant solution (fraction II), which was inactive, was removed and saved for the recombination experiments.

Step 2: removal of hemoglobin. The residue was washed with 100 ml of chilled 0.15 M KCl and centrifuged at 1,300 G for 60 minutes. The supernatant solution was removed by suction. The residue was transferred to a clean glass centrifuge bottle. In this manner the residue could be separated from the few unhemolyzed red cells which adhered to the glass surface. The residue was washed with 0.15 M KCl (10 to 15 times) until it appeared light yellow in color and was visually free of hemoglobin. On examination with a phase microscope, this residue (fraction III) consisted of stromal remnants and morphologically intact nuclei.

Step 3: extraction of enzyme with 0.15 M KCl. The residue (fraction III) was gelled by bringing the volume to 100 ml with chilled, de-ionized distilled water and by refrigerating the mixture overnight. Sufficient solid KCl was then added to make a 0.15 M KCl solution. This mixture was homogenized for 10 minutes in a Waring blender. The homogenate was centrifuged at 500 G for 30 minutes and the turbid, straw-colored, supernatant solution (fraction IV), was removed for assay of enzyme activity. The inactive precipitate was discarded.

An alternative method for preparing an active enzyme extract from the stromal-nuclei residue (fraction III) was as follows (Steps 3A and 4A).

Step 3A: preparation of acetone powder. Two hundred ml of acetone at − 60° C was added slowly, with continuous mixing, to 25 ml of washed, stromal-nuclei residue (fraction III). The mixture was centrifuged at 1,000 G for 10 minutes at − 20° C and the supernatant solution was discarded. After the precipitate was washed 3 times with 200 ml of acetone at − 60° C, it was suspended in cold acetone and filtered through Whatman no. 1 filter paper in a Büchner funnel. The precipitate was washed with 150 ml of ethyl ether at − 60° C and air-dried. The resulting powder (fraction V) was desiccated over concentrated sulfuric acid and stored at 4° C. The yield was 1.25 g.

Step 4A: extraction of the enzyme with Tween-20 and 0.15 M KCl. Acetone powder (fraction V), 2.25 g, was suspended at 4° C in 100 ml of 0.005 M Tris buffer, pH 7.8, and 0.4 ml of Tween-20 was added. The suspension was mixed intermittently for 30 minutes and allowed to stand overnight at 4° C. Solid KCl, 1.12 g, was added and the mixture was homogenized for 10 minutes in a precooled Waring blender. The homogenate was centrifuged at 500 G at 4° C for 30 minutes, and the slightly turbid, straw-colored supernatant solution (fraction VI) which contained the enzyme activity, was separated from the inactive precipitate. A preparation containing an equal concentration of Tween-20 but without any enzyme was inactive.

RESULTS

Studies on the various enzyme fractions

Recombination of heme synthetase fractions with fraction II. When either enzyme fraction IV or VI was assayed alone, and when fraction II was assayed alone, the amount of heme synthesized was small (Table I). However, the addition of fraction II to either enzyme fraction IV or VI resulted in considerable augmentation of heme synthesis. In eight experiments with different enzyme solutions, a two- to tenfold augmentation of enzyme activity was observed after the recombination of fraction II with either enzyme fraction IV or VI. Therefore, in subsequent experiments, the assay procedure was modified so that 1 ml of fraction II was added to each reaction mixture.

Specific activities of heme synthetase fractions. The results obtained in two typical fractionations are shown in Table II. When the specific activities of enzyme fractions IV and VI were compared in separate experiments with the specific activities of the whole blood hemolysates from which the enzyme fractions IV and VI had been

TABLE I

Effect of recombination of heme synthetase fractions with fraction II *

	Enzyme	Fraction II	Uptake Fe^{59}
	ml	*ml*	*%*
Fraction IV	5	0	2.3
	0	1	0.0
	5	1	6.7
Fraction VI	5	0	3.4
	0	1	0.3
	5	1	10.3

* Assay mixtures contained enzyme fractions and/or fraction II as indicated, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, and 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

TABLE II

The specific activities of heme synthetase fractions *

Fraction	Volume	Uptake Fe^{59}	Amt. of N assayed	Specific activity†	Total N	Total units	Yield
	ml	*%*	*mg*	*U*	*mg*		*%*
I Hemolysate	100	2.9	113.5	2.6	2,270	5,902	100
II Supernatant solution	125	0.0	10.1	0.0	1,262	0	0
IV 0.15 M KCl enzyme extract	95	17.0	1.0	1,700	19	32,300	540
I Hemolysate	180	7.6	79.42	10	2,859	27,446	100
II Supernatant solution	225	0.0	7.1	0	1,598	0	0
VI Tween-0.15 M KCl enzyme extract	48	20.8	1.64	1,268	16	20,288	73

* Assay mixtures contained 5 ml of hemolysate, 1 ml of fraction II or 5 ml of enzyme preparation (fractions IV or VI) with 1 ml of fraction II, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

† Specific activity (units) = 100 × per cent uptake Fe^{59} per 30 minutes per mg N.

prepared, a 654-fold and a 127-fold purification, respectively, were noted. The total units of enzyme in fractions IV and VI were high. A 5.4-fold increase in total enzyme units of fraction IV as compared to the units in the hemolysate (fraction I) was observed. This is probably because the measurement of enzyme activity in the hemolysate reflects, only in part, the amount of enzyme present, since the enzyme is located in the particulate fraction of the hemolysate (5). Although a further two-fold purification of fraction VI was achieved by a 35 to 45 per cent ammonium sulfate fractionation, the percentage yield was so low that this step offered little additional advantage in further purification.

TABLE III

The effect of temperature and dialysis *

Conditions	Per cent of original activity	
	Fraction II	Fraction IV
37° C	100	100
65° C	80	0
70° C	41	0
80° C	13	0
95° C	0	0
Undialyzed	100	100
Dialyzed	90	97

* Fractions II and IV were heated for 30 minutes or dialyzed for 24 hours against 1 L of 0.005 M Tris buffer, pH 7.8, with five changes of dialysate. Enzyme preparations contained either 1 ml of heated or dialyzed fraction II and 5 ml of fraction IV, or 5 ml of heated or dialyzed fraction IV and 1 ml of fraction II. In addition, assay mixtures contained 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, and 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

Properties of heme synthetase. The effect of temperature, dialysis, and storage at either 5° or − 5° C on the enzymic activity of fraction IV was studied. The enzyme was heat-labile and nondialyzable (Table III). When fraction IV was stored at either 5° or − 5° C for 2 weeks, there was complete loss of enzyme activity. However, enzyme fraction VI, prepared from an acetone powder (fraction V) which had been stored at 5° C for 4.5 months, was active. Unfortunately, it cannot be stated whether or not there was any decrease in activity during storage, since the acetone powder was not assayed with the same fraction II before as well as after storage.

Studies on the factor in fraction II

Properties. The effect of temperature, dialysis, and storage at either 5° or − 5° C on the factor in fraction II was studied. This factor was heat-labile and nondialyzable (Table III). After storage for 3 months at either 5° or − 5° C, the factor in fraction II was still effective in augmenting heme synthesis.

Effect of globin. The preliminary characterization described above suggested that the factor in fraction II was a protein. Therefore, a series of

TABLE IV

The effect of globin *

Enzyme (chicken) fraction IV	Preparations added	Amt.	Uptake Fe^{59}
ml			%
0	Fraction II (chicken)	1 ml	0.0
5	None		0.7
5	Fraction II (chicken)	1 ml	4.5
5	Fraction II (human)	1 ml	4.3
5	Serum (chicken)	1 ml	0.2
5	Serum (human)	1 ml	0.0
5	Albumin (human)	0.16 μmole	0.3
5	Gamma globulin (human)	0.16 μmole	0.7
5	Transferrin (human)	0.16 μmole	0.2
0	Globin (chicken)	0.16 μmole	0.2
5	Globin (chicken)	0.16 μmole	2.1
0	Globin (human Hb A)	0.16 μmole	0.2
5	Globin (human Hb A)	0.16 μmole	1.9
5	Globin (human Hb SC)	0.16 μmole	2.3
5	Globin (human Hb G)	0.16 μmole	2.6

* Assay mixtures contained enzyme fraction IV and/or protein preparations as indicated, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, and 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

assays with enzyme fraction IV was performed in which chicken and human sera, purified human serum fractions, and chicken and human globins were substituted for fraction II (Table IV). Augmentation of enzyme activity occurred with globin prepared from chicken hemoglobin and human hemoglobins A, SC, and G; however, no effect could be demonstrated with whole serum or with albumin, γ-globulin or transferrin.

The effect of globin concentration on heme synthesis was studied in a separate experiment. Various amounts of lyophilized chicken globin were dissolved in 1 ml of de-ionized distilled water, added to 5-ml aliquots of enzyme fraction IV, and the mixtures were assayed (Table V). Maximal synthesis occurred when 1 ml of a solution containing 0.16 μmole globin was substituted for 1 ml of fraction II. At higher globin concentrations there was less augmentation of enzyme activity. Control mixtures of globin, assayed with 5 ml of 0.15 M KCl instead of enzyme, gave less than a 0.4 per cent uptake of Fe^{59}. All of the reaction mixtures remained at pH 7.8 during incubation.

One-ml aliquots, containing 0.16 μmole of an active globin preparation, which had been lyophilized and stored at 4° C, were assayed every 3 weeks with 5 ml of fresh enzyme fraction IV. After storage for 3 weeks, the globin was only 15 per cent as effective in stimulating heme synthesis as the fresh globin. After storage for 6 weeks, the globin failed to stimulate heme synthesis.

Characterization of the reaction product. The observation that globin could be substituted for the factor in fraction II suggested that its role in this reaction might be to bind with the newly synthesized heme to form hemoglobin. To determine whether hemoglobin or heme was the end-product of the reaction, the radioactivity of hemoglobin, isolated by starch block electrophoresis was studied. Reaction mixtures (Table VI), which had been allowed to proceed for 240 minutes to obtain maximal incorporation of label, were centrifuged at 10,000 G, and dialyzed against 0.05 M barbital buffer, pH 8.6, or 0.05 M phosphate buffer, pH 6.8. Electrophoresis of 0.3-ml aliquots was then carried out at pH 8.6 and 6.8, respectively.

TABLE V

The effect of globin concentration on heme synthesis *

Enzyme fraction IV (chicken)	Preparations added		Per cent uptake Fe^{59}
	Fraction II (chicken)	Globin (chicken)	
ml	*ml*	*μmoles*	
0	1		0.0
5	0		2.6
5	1		8.2
5		1×10^{-3}	2.8
5		1×10^{-2}	3.3
5		5.6×10^{-2}	5.5
5		1×10^{-1}	6.5
5		1.6×10^{-1}	8.5
5		3.2×10^{-1}	7.7
5		5.6×10^{-1}	5.6
5		1	3.1

* Assay mixtures contained 5 ml of enzyme fraction IV, 1 ml of a globin chloride solution, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, and 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

TABLE VI

Recovery of radioactive hemin in the hemoglobin fraction *

Electrophoresis	Total hemin radioactivity		Per cent of hemin radioactivity in Hb
	Before	After	
	cpm $\times 10^{-3}$		
pH 8.6	51.5	51.7	100
pH 6.8	54.4	40.2	74

* Assay mixtures contained 5 ml of enzyme fraction IV, 1 ml of fraction II, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine, and 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. The incubations were for 240 minutes at 37° C, and the reaction was stopped with 1 ml of 0.1 M potassium cyanide. Starch block electrophoresis was performed in 0.05 M barbital buffer, pH 8.6, and in 0.05 M phosphate buffer, pH 6.8, at 400 v for 4 and 16 hours, respectively.

The hemoglobin, when electrophoresed at pH 8.6 for 4 hours, remained at the origin with a leading edge toward the anode. When electrophoresed at pH 6.8 for 16 hours, the hemoglobin separated into three bands which moved toward the cathode. When a solution of heme was electrophoresed at either pH, the heme migrated rapidly to the anode. After electrophoresis, the starch blocks were cut into five sections so that one section contained all the visible hemoglobin. The samples were eluted with two buffer washes of 7 ml total volume. Carrier hemoglobin was added to each eluate; the heme was crystallized in order to remove the non-heme radioiron, the radioactivity of the hemin was counted, and the total hemin radioactivity was determined.

One hundred per cent of the radioactive heme was recovered with the hemoglobin (Table VI) after electrophoresis at pH 8.6. Seventy-four per cent of the radioactive heme was recovered with the hemoglobin after electrophoresis at pH 6.8. None of the other sections of the starch block contained significant radioactivity.

In a similar experiment, the radioactivity of hemoglobin isolated by chromatography on an IRC-50 column was investigated. To eliminate the possibility that the heme was bound to a protein other than hemoglobin but with the same isoelectric point, a hemoglobin SC hemolysate (18) was used instead of chicken fraction II in the reaction mixture. The reaction mixture was chromatographed and the minor hemoglobin components (14) and the major hemoglobin S and hemoglobin C components were separated. As the column was developed, the hemoglobin concentration, as measured by absorbance at 415 mμ, and the radioactivity were determined in each milliliter of eluate. The amount of radioactive heme present in pooled samples of from 1 to 4 ml, depending on the radioactivity, was then determined.

When the radioactivity of the heme and the concentration of hemoglobin were plotted on the same axis, the peaks of radioactivity and hemoglobin coincided (Figure 1). The specific activities, expressed as the hemin radioactivity (cpm) per unit of absorbancy for each hemoglobin fraction, were as follows: minor hemoglobin components, 158 cpm; major hemoglobin S, 148 cpm; and major hemoglobin C, 173 cpm. Similar labeling of the minor and major hemoglobin components occurred in an experiment in which a hemoglobin A hemolysate was used.

DISCUSSION

In the experiments presented (Table II), when the specific activities of enzyme fractions IV and VI were compared with those of the hemolysates from which they had been prepared, a 654-fold and a 127-fold purification, respectively, were noted. Similar preparations of fraction VI from different hemolysates have varied from 50- to 900-fold in the purification of enzyme from the hemolysate. Fraction IV, like previously described enzyme preparations (5–7), was unstable. However, enzyme stored as an acetone powder (fraction V) was stable. This suggests that the preparation of an acetone powder (fraction V) would be a preferable procedure in further purification studies.

The purification of the heme synthetase system was facilitated by the observation that a heat-labile, nondialyzable, stable factor present in the supernant solution (fraction II) of the original hemolysate was required, in addition to the enzyme, for maximal synthesis of heme. Although human nonreticulated cells are not capable of synthesizing heme (3), fraction II, prepared from human cells, was as effective as chicken fraction II in augmenting the activity of purified heme synthetase. Similar observations have been made by Minakami, Yoneyama and Yoshikawa (19). Rabinovitz and Olson (20) have reported that the incorporation of iron into hemoglobin by a rabbit reticulocyte microsomal system requires a soluble fraction.

The effect of globin accounts in part, if not entirely, for the effect of fraction II on the synthesis of heme. Globin was prepared five times by the method of Rossi-Fanelli, Antonini and Caputo (12), and four of the preparations stimulated heme synthesis. With two of these preparations the incorporation of radioiron into heme was as great in the presence of optimal amounts of globin as in the presence of fraction II. When globin was prepared five times by the method of Anson and Mirsky (21), four of the preparations also stimulated heme synthesis. With none of these preparations was the incorporation of radioiron into heme as great as in the presence of fraction II. This variability in the effectiveness of different preparations of globin to stimulate heme synthesis and

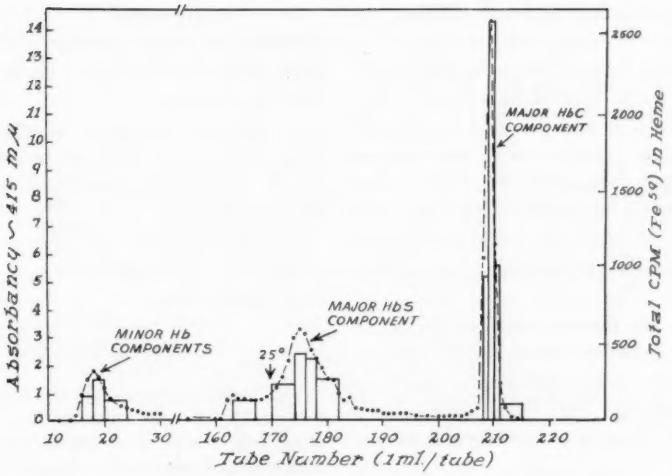

FIG. 1. THE CHROMATOGRAPHIC SEPARATION OF RADIOACTIVE HEMOGLOBINS S AND C. The radioactivity of the heme (columns), expressed as total cpm in the heme, and the concentration of hemoglobin (broken line), expressed as absorbancy at 415 mμ, are compared. The assay mixture contained 5 ml of enzyme fraction VI, 1 ml of a hemoglobin SC hemolysate, 0.5 μmole protoporphyrin, 0.13 μc ferrous (Fe^{59}) citrate, 10 μmoles cysteine and 1,000 μmoles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. The incubation was for 240 minutes at 37° C, and the reaction was stopped with 1 ml of 0.1 M potassium cyanide. There was 55.1 per cent uptake of Fe^{59}. In a similar assay mixture, which did not contain enzyme, there was 0.6 per cent uptake of Fe^{59}. Chromatography was on a 1 × 35 cm IRC-50 column which was developed at 5° C with phosphate buffer, pH 7 (Developer no. 2). The buffer was 0.0625 M in sodium ion. At tube no. 170, the column was warmed to 25° C. The distortion seen in the hemoglobin S peak was produced by the emergence of some of this hemoglobin before the column warmed to 25° C.

the failure to observe an effect with stored globin preparations are consistent with the known instability of purified globin preparations (*22*) and may explain why other investigators have not observed an effect of globin on heme synthesis (*23*).

The recovery of most, if not all, of the radioactivity in the heme from the hemoglobin isolated by electrophoresis or chromatography can be taken as evidence that the end-product of the reaction was hemoglobin and not free heme. In this regard it is interesting that the observed (Table V) optimal ratio of globin concentration (0.16 μmole per 10 ml reaction mixture) to protoporphyrin concentration (0.5 μmole per 10 ml reaction mixture) was 1.2 : 4. This ratio is in good agreement with the expected 1 : 4 ratio, i.e., four heme groups per molecule of hemoglobin. Furthermore, when different hemoglobin components from a reaction mixture were separated by chromatography, it was found that the labeling of the hemoglobin was of similar specific activity whether globin from hemoglobins A, S, C or from minor components was available. Similar observations were made by Minakami and associates (19) when the enzymic incorporation of radioiron into horse and duck hemoglobins was studied. The abnormal hemoglobins S, C and G have been shown to differ from hemoglobin A in their primary structure (*24, 25*); however, globins prepared from these hemoglobins were as effective as chicken globin in stimulating heme synthesis.

Although the nonenzymic formation of heme has been observed at neutral pH in the presence of protoporphyrin, iron, globin and a detergent (*26*), we have not been able to detect such a reaction under the conditions of our experiments. In the

present studies, globin, in addition to cysteine, or reduced glutathione, was shown to be required for maximal synthesis of heme from iron and protoporphyrin by the heme synthetase system. The observed effect of globin and the lack of effect of other proteins on the synthesis of heme suggests that the globin effect is specific. However, since the enzymic synthesis of heme is probably the same in all tissues (*27–29*), one might expect that other heme apoproteins will show an effect similar to that of globin.

The present studies do not elucidate the exact mechanism of hemoglobin formation from iron, protoporphyrin and globin. The heme, which is synthesized enzymically, may bind with globin (30), drawing the reaction to the right as hemoglobin is formed:

$$\text{Fe} + \text{protoporphyrin} \underset{\text{heme synthetase}}{\rightleftharpoons} [\text{heme}] + \text{globin} \rightleftharpoons \text{Hb.}$$

Presumptive evidence in favor of this mechanism is the recent demonstration by Rossi-Fanelli and Antonini (31) that the heme groups of heme proteins exist in equilibrium with free heme and can exchange with other apoproteins. On the other hand, the globin may act in the formation of a protoporphyrin-globin complex (PP-G) prior to the enzymic incorporation of iron (32):

$$\text{protoporphyrin} + \text{globin} \rightleftharpoons [\text{PP-G}] + \text{Fe} \underset{\text{heme synthetase}}{\rightleftharpoons} \text{Hb.}$$

Elucidation of the mechanism of these reactions will have to await the isolation of the pure enzyme and subsequent kinetic studies.

SUMMARY

1. The formation of hemoglobin from iron, protoporphyrin and globin was studied in a chicken hemolysate system. Heme synthesis was measured as the per cent incorporation of added radioiron into heme.

2. The heme synthetase enzyme system was purified several hundredfold. An acetone powder of a purified enzyme preparation was stable for 4.5 months at 5° C.

3. A factor present in the supernatant solution (fraction II) from a chicken or human hemolysate was required for maximal incorporation of radioiron into heme.

4. The factor present in fraction II could be replaced by optimal concentrations of chicken globin. A similar effect was observed with human globin.

5. The end-product of the reaction, as characterized by electrophoresis and chromatography, was hemoglobin.

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STUDIES ON THE RESPONSE OF THE TRANSPLANTED KIDNEY AND THE TRANSPLANTED ADRENAL GLAND TO THORACIC INFERIOR VENA CAVAL CONSTRICTION *

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The mechanisms involved in the marked sodium retention observed in such clinical states as congestive heart failure and advanced portal cirrhosis continue to pose a challenging problem. The role of increased renal venous pressure and of efferent nervous pathways to the kidney in effecting sodium retention, and the role of the adrenal nerves in the hypersecretion of aldosterone observed in such states demands further clarification. In investigating the role of increased venous pressure in experimental ascites produced by thoracic inferior vena caval constriction in the dog, Whelan, McCoord and Schilling (1) have reported that the kidney transplanted to the neck failed to exhibit the same degree of sodium retention as the normal kidney following thoracic caval constriction; however, the fact that one of their four experimental animals did show virtually complete sodium retention following caval constriction suggests the kidney removed from the congested circulation may respond just as the normally located kidney does to thoracic inferior vena caval constriction. In studying the mechanisms of sodium retention in dogs with experimental congestive heart failure, Barger, Muldowney and Liebowitz (2) have suggested that increased sympathetic tone of the renal nerves is an important factor in severe chronic sodium retention; other investigators (3–6) have, however, concluded that complete renal denervation has no consistent effect on sodium chloride excretion. In an investigation of the role of the adrenal nerves in the control of aldosterone secretion, Fleming and Farrell (7) have pointed out that the acutely transplanted adrenal released aldosterone at a normal or slightly elevated rate. Denton, Goding and Wright (8) have demonstrated that the adrenal chronically transplanted to the neck in sheep increased the rate of aldosterone secretion in response to sodium depletion. Quantitative measurements of aldosterone secretion by the transplanted adrenal gland in animals with chronic sodium retention have, however, not before been reported.

In the present study, two experiments have been performed. The first experiment was designed to investigate the role played by the renal nerves and by increased renal venous pressure in the marked sodium retention observed after thoracic inferior vena caval constriction in the dog. The second experiment was aimed to determine whether efferent nervous pathways to the adrenal are essential to the hypersecretion of aldosterone observed in the dog after thoracic caval constriction and superimposed acute hemorrhage. In the first experiment, studies have been made of the response of the animal with a single transplanted kidney to thoracic inferior vena caval constriction. In the second experiment, the response of the animal with a single transplanted adrenal gland to chronic thoracic inferior vena caval constriction and to superimposed acute hemorrhage has been investigated. The secretion rates of aldosterone and corticosterone have been determined after thoracic caval constriction, and chronic electrolyte balance studies have been carried out in both experiments.

MATERIAL AND METHODS

Experiments were performed on mongrel dogs weighing 15 to 20 kg. In Experiment I (6 dogs), the left kidney and adrenal were transplanted to the neck by the procedure described by Levy and Blalock (9). Approximately one week later, right nephrectomy was performed.

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One week after nephrectomy the thoracic inferior vena cava was constricted. In 3 of these animals, after two weeks of observations, the right adrenolumbar vein was cannulated under sodium pentobarbital anesthesia, and the adrenal venous effluent was collected.

In Experiment II (3 dogs), initially the left kidney and adrenal were transplanted to the neck. Approximately one week later, the left (cervical) kidney was carefully dissected out and excised, with preservation of the renal artery and vein; at the same time the right (abdominal) adrenal was removed. Two to three weeks following the latter procedure, thoracic inferior vena caval constriction was performed. One to two weeks later, the left external jugular vein was cannulated under sodium pentobarbital anesthesia, and adrenal venous blood was obtained both before and 30 to 90 minutes after removal of 250 ml of blood via the femoral vein.

Each animal was kept in a metabolic cage throughout the experiments and received (by forced feeding when necessary) a synthetic diet containing 60 mEq sodium and 18 mEq potassium per day. All animals were allowed water *ad libitum*. In Experiment I urine dripped from the open end of the ureter, which was transplanted to the surface of the neck, to the collecting pan of the metabolic cage. This pan was washed with 500 ml of distilled water immediately prior to removal of the 24-hour collection jar. Urinary and fecal sodium and potassium excretion was determined throughout the experiments, and in Experiment II plasma sodium and potassium concentrations were measured at frequent intervals by flame photometry. Inferior vena caval pressures were determined with a water manometer. Arterial pressures were measured by direct puncture of the femoral artery; a Statham strain gage and a Sanborn recording system were used for recording of pressures. Ascites was removed by paracentesis at frequent intervals.

Adrenal venous aldosterone and corticosterone concentrations were determined by the double isotope derivative technique (10), and secretion rates of these steroids were calculated by a method described previously (11). In one animal, urinary aldosterone was determined by a modification of the double isotope technique with the addition of a fourth chromatographic step to provide better purification (12). Secretion rates of 17,21-dihydroxy-20-ketosteroids were determined by a modification of the Porter-Silber method as described by Peterson, Karrer and Guerra (13).

For pathological studies, transplanted kidneys and adrenal glands were fixed in 10 per cent formalin. Following fixation of the adrenal gland, extraneous tissues were carefully removed and the adrenals were weighed. Frozen microtome sections of adrenal glands were stained with Sudan IV. Paraffin sections of adrenal and renal tissue were stained with hematoxylin and eosin, and with azure-eosin (pH 4.5).

RESULTS

Experiment I: Effects of chronic constriction of the thoracic inferior vena cava following transplant of the left kidney to the neck and right nephrectomy. Following transplantation of the left kidney and adrenal gland to the neck and right nephrectomy, all six animals achieved sodium balance within two days. The average daily urinary

TABLE I

Effects of thoracic caval constriction on sodium and potassium excretion in dogs following transplantation of the left kidney to the neck and right nephrectomy

Dog no.	Before constriction					After constriction				
	Na excretion		K excretion		Fecal Na/K	Na excretion		K excretion		Fecal Na/K
	Urine	Feces	Urine	Feces		Urine	Feces	Urine	Feces	
	mEq/day		*mEq/day*			*mEq/day*		*mEq/day*		
1	43.1 [6]†	3.5	18.7	0.9	3.9	2.5* [7]	1.6	13.8	2.2	0.7
2	48.2 [6]	7.0	18.8	1.4	5.0	1.6 [9]	0.4	15.5	5.2	0.1
3	31.0 [7]	1.6	13.1	0.7	2.3	1.9 [7]	0.8	17.4	4.4	0.2
4	47.1 [6]	5.4	19.1	1.4	3.9	3.9 [9]	3.4	14.4	7.6	0.5
5	38.8 [6]	1.3	20.8	2.2		0.8 [6]		5.4		
6	31.8 [1]		17.7			1.5 [5]		14.8		

* The values presented for sodium and potassium excretion after thoracic caval constriction in Dog 1 represent the average values for the first 7 days after caval constriction (see Figure 2).

† The numbers in brackets indicate the number of days that measurements were made for urinary and fecal electrolyte excretion. Values represent averages for each period.

TABLE II

Aldosterone secretion by the normally located right adrenal following transplantation of the left kidney and adrenal to the neck, right nephrectomy and thoracic inferior vena caval constriction

Dog no.	Aldosterone secretion	Corticosterone secretion
	μg/min	*μg/min*
3	0.090	3.31
	0.085	5.08
5	0.054	1.78
	0.044	0.79
6	0.147	1.78
	0.132	1.97
Normal dogs stressed by laparotomy (n = 10)	0.024 ± 0.017	2.13 ± 0.65
Dogs with thoracic caval constriction stressed by laparatomy (n = 16)	0.135 ± 0.056	3.11 ± 1.78

volume produced by the cervical kidney ranged from 237 to 474 ml in the six dogs. All animals appeared in excellent health at the time of thoracic caval constriction. After caval constriction, inferior vena caval pressures increased from an average normal value of 51 mm of water (14) to the range of 178 to 320 mm of water; virtually complete sodium retention was observed in every animal, and urinary potassium excretion was decreased in five of the six dogs (Table I and Figures 1 and 2). The low fecal sodium/potassium ratio resulted in each animal from an increase in fecal potassium as well as from a decrease in fecal sodium excretion. Aldosterone secretion by the normally located right adrenal gland, determined two weeks after thoracic inferior vena caval constriction, was elevated in the three animals in which determinations were made. In each of these three dogs, aldosterone secretion was in the range previously observed in dogs subjected to thoracic inferior vena caval constriction (Table II) and was adequate to account for the observed sodium retention. Corticosterone secretion was in the same range as that of normal animals stressed by laparotomy and adrenolumbar vein cannulation (Table II). Urinary aldosterone excretion was very low (less than 0.1 μg per day) prior to caval constriction and increased strikingly to a level of 1.65 μg per day after caval constriction (Figure 1). Arterial pressure remained at the normal level throughout the experiment in all animals.

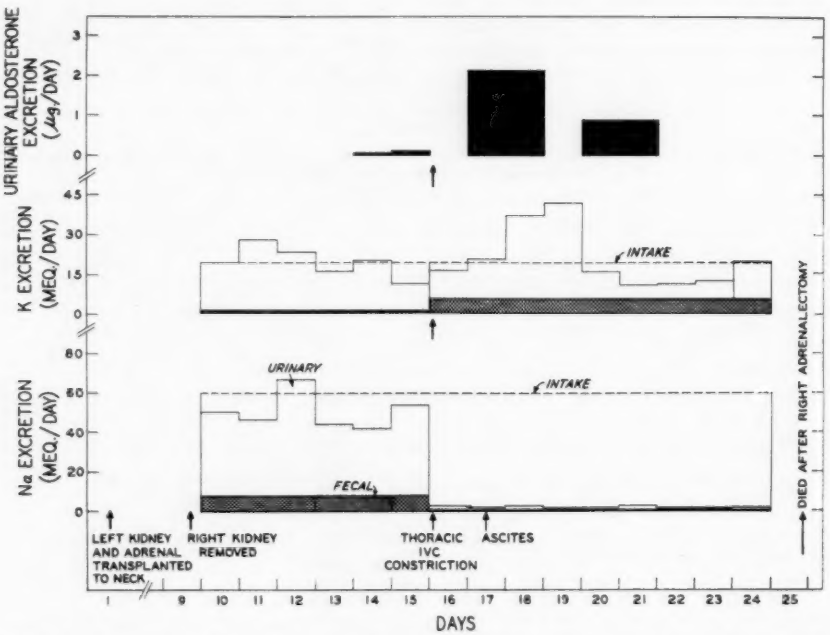

FIG. 1. EFFECTS OF CHRONIC THORACIC INFERIOR VENA CAVAL CONSTRICTION FOLLOWING TRANSPLANT OF LEFT KIDNEY AND ADRENAL TO THE NECK AND RIGHT NEPHRECTOMY IN DOG 2, TABLE I.

In Dog 3, the right (abdominal) adrenal gland was removed after collection of adrenal venous blood. Metabolic studies were continued during the following week, at a time when neither the kidney nor the adrenal gland was in the normal abdominal position. During this period, the marked sodium retention continued (Table III).

In one dog (Figure 2), thoracic inferior vena caval pressure, which was 255 mm of water on the sixth day after caval constriction, fell to 210 on the twelfth postoperative day, presumably secondary to venous collateralization. Concomitant with the fall in vena caval pressure, a marked increase in sodium excretion occurred. At this time, reconstriction of the thoracic inferior vena cava resulted in a rise in venous pressure to 320 mm of water, and almost complete sodium retention again occurred.

Pathological examination of the transplanted kidneys was performed in three cases (Dogs 1, 3 and 5). Each of these kidneys appeared grossly normal. On microscopic examination, focal areas of necrosis were observed, but in no instance did the necrosis involve more than 10 per cent of the renal parenchyma.

TABLE III

Effects of thoracic caval constriction and subsequent removal of the right (abdominal) adrenal gland on sodium and potassium excretion following transplant of left kidney and adrenal gland to the neck and right nephrectomy (Dog 3, Table I)

Dog 3		Before caval constriction	After caval constriction	After removal of right (abdominal) adrenal
Na excretion (mEq/day)	Urine	31.0 [7]*	1.9 [7]	1.0 [5]
	Feces	1.6	0.8	0.6
K excretion (mEq/day)	Urine	13.1	17.4	14.0
	Feces	0.7	4.4	2.0

* The number in brackets indicates the number of days in which measurements were made for urinary and fecal electrolyte excretion. Values represent averages for each period.

Experiment II: Effects of chronic thoracic inferior vena caval constriction following transplant of the left adrenal and kidney to the neck, right

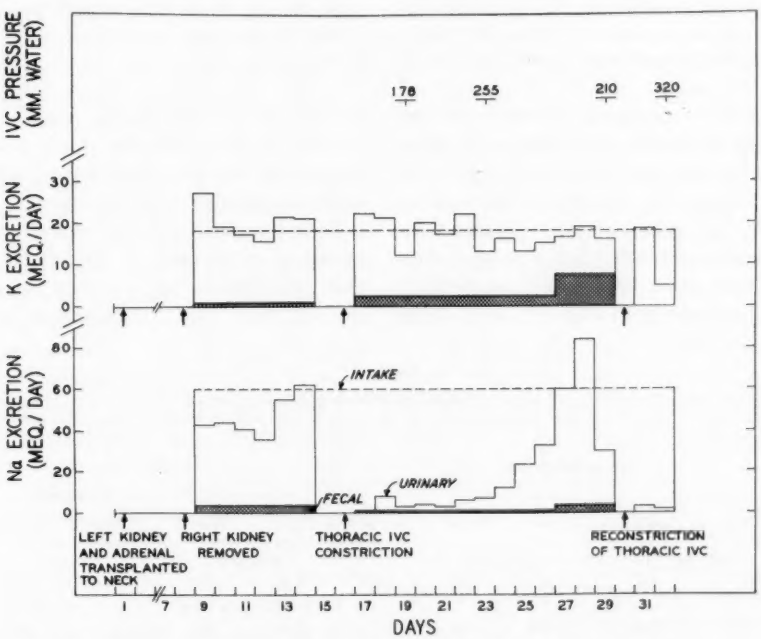

FIG. 2. EFFECTS OF CHRONIC THORACIC INFERIOR VENA CAVAL CONSTRICTION FOLLOWING TRANSPLANT OF LEFT KIDNEY AND ADRENAL TO THE NECK AND RIGHT NEPHRECTOMY IN DOG 1, TABLE I. Note the natriuresis concurrent with the fall in inferior vena caval (IVC) pressure and the almost complete sodium retention following retightening of caval ligature.

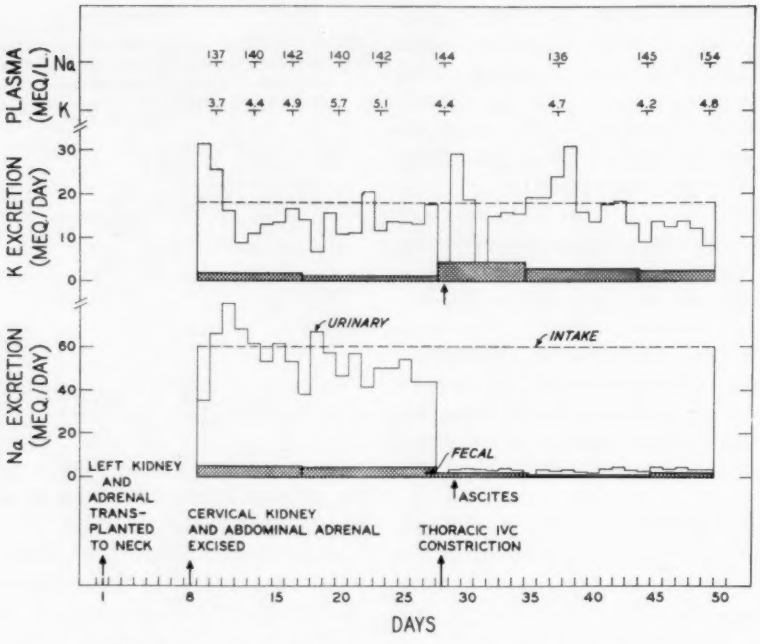

FIG. 3. EFFECTS OF CHRONIC THORACIC INFERIOR VENA CAVAL CONSTRICTION FOLLOWING TRANSPLANT OF LEFT ADRENAL AND KIDNEY TO THE NECK, RIGHT ADRENALECTOMY AND CERVICAL NEPHRECTOMY IN DOG 7, TABLE IV.

adrenalectomy and left (cervical) nephrectomy. After right adrenalectomy and cervical nephrectomy, sodium balance was rapidly achieved by all animals (see Figure 3). Plasma sodium and potassium levels and mean arterial pressures remained within normal limits. Subsequent to thoracic inferior vena caval constriction, marked sodium retention occurred (Table IV) and ascites accumulated rapidly in all dogs. The fecal sodium/potassium ratio decreased, and urinary potassium excretion declined in two of the three animals following caval constriction.

In two animals, adrenal venous blood was obtained by cannulation of the left external jugular vein proximal to the site of its anastomosis with the left renal vein. Aldosterone, corticosterone,

TABLE IV

Effects of thoracic inferior vena caval constriction on sodium and potassium excretion in dogs following transplantation of the left adrenal and kidney to the neck, right adrenalectomy and cervical nephrectomy

Dog no.	Before constriction					After constriction				
	Na excretion		K excretion		Fecal Na/K	Na excretion		K excretion		Fecal Na/K
	Urine	Feces	Urine	Feces		Urine	Feces	Urine	Feces	
	mEq/day		*mEq/day*			*mEq/day*		*mEq/day*		
7	50.7 [19]*	4.7	17.6	1.6	3.0	1.2 [21]	1.8	14.8	3.1	0.6
8	42.1 [17]	7.8	15.0	1.6	4.7	0.7 [14]	0.9	9.7	2.0	0.5
9	50.3 [13]		18.8			2.1 [12]		18.0		

* The numbers in brackets indicate the number of days on which measurements were made for urinary and fecal electrolyte excretion. Values represent averages for each period.

TABLE V

Steroid secretion by the cervical adrenal gland in dogs with thoracic caval constriction before and 30 to 90 minutes after hemorrhage

Dog no.	Time	Aldosterone secretion	Corticosterone secretion	Porter-Silber chromogen secretion
	min	*μg/min*	*μg/min*	*μg/min*
8	0	0.078	0.23	0.39
	10	0.049	0.06	3.91
	30 → bled			
	70	0.077	0.84	5.87
	100	0.103	1.04	6.65
	120	0.103	0.98	
9	0	0.005	0.15	0.90
	10	0.006	0.17	0.86
	20 → bled			
	60	0.036	0.85	1.99
	70	0.033	0.60	3.82
Conscious unstressed normal dogs (n = 5)		0.006 ± 0.002	0.29 ± 0.20	0.80 ± 0.20
Normal dogs stressed by laparotomy		0.024 ± 0.017 (n = 10)	2.13 ± 0.65 (n = 10)	7.34 ± 2.69 (n = 7)

and Porter-Silber chromogen secretion rates were determined. The results are presented in Table V. In one animal, the rate of aldosterone secretion was clearly elevated. In the other dog, aldosterone secretion appeared to be low; this was probably because only a fraction of the adrenal venous effluent was returning via the left external jugular vein. Corticosterone and Porter-Silber chromogen secretion rates were low in both dogs; the values were in the same range as those observed in unstressed conscious normal dogs with chronic adrenal venous catheters (12), and were far below the secretion rates of normal dogs stressed by laparotomy (15).

Following collection of the control samples of adrenal venous blood, both dogs were subjected to acute hemorrhage. An increase in the secretion rates of aldosterone, corticosterone, and Porter-Silber reacting chromogens occurred within 40 to 70 minutes after the blood loss (Table V). It is noteworthy that even in Dog 8, in which the control secretion rate of aldosterone was elevated as the result of chronic inferior vena caval constriction, a further increase in aldosterone secretion rate occurred following hemorrhage. The increments in secretion rates of corticosterone (fourfold) and of Porter-Silber chromogens (two- to fourfold) were similar for each of the two animals.

Each of the three transplanted adrenal glands in Experiment II was examined for pathological changes. The transplanted adrenals appeared normal grossly and ranged in weight from 1.0 to 1.9 g. On microscopic examination, small areas of necrosis, involving both cortex and medulla, were present in each gland; the necrosis did not involve more than 25 per cent of the adrenal parenchyma in any instance. Normal zonal adrenocortical architecture was observed. The zona glomerulosa was easily distinguished from the outer and inner fasciculata and juxtamedullary regions. In areas where only a portion of the thickness of the cortex remained, the zona glomerulosa was most often preserved.

DISCUSSION

All animals in Experiment I remained in good clinical condition following transplantation of the left kidney to the neck and right nephrectomy. This observation is in accord with the work of Dempster (16), who noted survival of dogs in apparent good health for over a year after unilateral nephrectomy and transplantation of the remaining

kidney to the neck. However, the marked polyuria, which was observed following removal of the normal kidney in the cases reported by Dempster, failed to occur in any of the present experimental animals. Urinary volume from the neck kidney after uninephrectomy was not significantly different from the normal total urinary output.

Renal hemodynamic function studies were not performed in the present experiments. On the basis of the observations of Bricker, Straffon, Mahoney and Merrill (3), the possibility must be considered that both renal plasma flow and glomerular filtration rate (GFR) in the cervical kidney were reduced. In each animal, however, the cervical kidney consistently demonstrated the ability to maintain normal sodium and potassium balances during the period prior to thoracic inferior vena caval constriction. In one animal the brisk natriuresis which accompanied the fall in venous pressure indicated that the cervical kidney was capable of excreting sodium at a rate adequate to produce a negative sodium balance. Hence, it appears that the renal hemodynamic alterations attendant upon transplantation of the kidney to the neck played no essential role in the sodium retention observed after caval constriction in the present experimental animals. The observed hypersecretion of aldosterone in the presence of elevated venous pressure secondary to thoracic caval constriction provides an adequate explanation for the sodium retention (17), but a possible contributory influence of a decreased GFR cannot be excluded.

In an investigation of the role of increased renal venous pressure in the sodium retention produced by thoracic caval constriction, Whelan and associates (1) performed experiments similar to those of the present study, although in two of their four dogs, thoracic caval constriction was carried out prior to removal of the abdominal kidney. Their results, however, were inconclusive, since one animal failed to show a positive sodium balance after thoracic caval constriction, and varying degrees of sodium retention were observed in the other three dogs; only one of their four animals showed the virtually complete sodium retention which was characteristic of all the animals described in the present study. Although Whelan and co-workers attributed their results to a diminished ability of the transplanted kidney to retain sodium, it appears more likely that the elevation in inferior vena caval pressure was inadequate in their experimental dogs. The present observations demonstrate that the kidney transplanted to the neck exhibited the same marked sodium retention as do normally located kidneys following thoracic inferior vena caval constriction (18).

In investigating the role of the renal nerves in the sodium retention of congestive heart failure, Barger and associates (2) have pointed out that the infusion of 10 μg of aldosterone into the renal artery of a normal dog failed to cause a unilateral antinatriuresis, whereas a much smaller dose of aldosterone injected into the renal artery of an animal with experimental tricuspid insufficiency exerted a definite antinatriuretic effect. They have further observed that norepinephrine injected into the renal artery of a normal dog caused a unilateral decrease in renal sodium excretion, whereas Dibenzyline (phenoxybenzamine hydrochloride) injected into the renal artery of a dog with experimental tricuspid insufficiency caused marked natriuresis. On this basis, Barger and co-workers have postulated that the renal nerves or the renal response to circulating norepinephrine play an important role in the severe sodium retention of congestive heart failure. The almost complete sodium retention in the present experimental dogs with denervated, transplanted kidneys demonstrates that the renal nerves are not essential to the profound chronic sodium retention which follows thoracic inferior vena caval constriction. Our present experimental results are in accord with those and Bricker and associates (3) who found, in studying the function of the kidney transplanted to the femoral region in the dog, that the "complete denervation occurring during the transplantation procedure did not result in any consistent abnormality in the tubular transport of sodium chloride."

The results obtained from Experiment II, in which the left adrenal gland was transplanted to the neck and the right adrenal was excised, confirm the observations of Levy and Blalock (9) that the transplanted adrenal gland in the dog secretes adequate steroid to maintain the animal in excellent health. Actual measurements of the basal secretion rates of steroids in the present experiment further demonstrate that the secretion rates of corticosterone and Porter-Silber chromogens by the transplanted adrenal were in the same

range as those of the abdominal adrenal of normal unstressed conscious dogs (12).

In investigating the role played by the adrenal nerves in the regulation of aldosterone secretion, Fleming and Farrell (7) acutely transplanted the adrenal gland of a donor animal to the thigh of a recipient dog. They measured the aldosterone and cortisol output by the transplanted adrenal and found the secretion rates to be as high as, or higher than, the secretion rates by the normal adrenal gland of the anesthetized dog stressed by laparotomy. However, they did not subject the recipient animal to any stimulation to determine whether the transplanted adrenal would respond to an appropriate stimulus with an increase in aldosterone secretion. In their extensive studies on the adrenal gland transplanted to a cervical pouch in sheep, Denton and associates (8) have shown that the transplanted gland, in response to sodium depletion, exhibited at least a fivefold increase in secretion rate of aldosterone. The present experimental data show that the transplanted adrenal gland not only responded to thoracic caval constriction by hypersecretion of aldosterone, but also responded to superimposed acute hemorrhage by a further increase in rate of aldosterone secretion. As in the studies of Denton and associates, the hypersecretion occurred after complete adrenal denervation, a finding which supports the thesis of Yankopoulos, Davis, Kliman and Peterson (15) that a humoral factor, an aldosterone-stimulating hormone, provides the immediate stimulus to hypersecretion of aldosterone in experimental secondary hyperaldosteronism.

The present experiments indicate that acute hemorrhage stimulates the secretion not only of aldosterone, but also of other corticosteroids. The increase in secretion of corticosterone and Porter-Silber chromogens demonstrates that increased ACTH release is an important part of the response of the animal to hemorrhage. In hypophysectomized dogs, corticosterone and Porter-Silber chromogen output either failed to increase or increased only slightly following hemorrhage (personal observations). It has previously been recognized that acute hemorrhage causes an increase in the secretion rate of aldosterone in both the normal (19) and the hypophysectomized (20, 21) dog, and it has been assumed that the increase in normal animals is mediated via a volume receptor system activated by the decreased blood volume (22). The fact that acute hemorrhage causes also an increased release of ACTH in the normal dog has previously been obscured because the animals studied have been subjected to the stress of laparotomy and adrenolumbar vein cannulation (19), and consequently, control secretion rates of corticosteroids have approached maximal levels. In the two animals here described, the cervical adrenal venous effluent was collected after a simple skin incision, so that the control corticosterone and Porter-Silber chromogen secretion rates were very low; therefore, increased ACTH release in response to hemorrhage was clearly demonstrable by the striking increase in corticosteroid output.

SUMMARY AND CONCLUSIONS

The response of a single transplanted cervical kidney to chronic thoracic inferior vena caval constriction has been investigated in six dogs. In each animal, the transplanted kidney responded to caval constriction in essentially the same manner as a normally located kidney, with virtually complete sodium retention. The response of a single transplanted cervical adrenal gland to chronic thoracic caval constriction and to superimposed acute hemorrhage has been studied in three dogs. In each animal, the transplanted adrenal gland secreted adequate aldosterone to effect the almost complete sodium retention and the very low fecal sodium/potassium ratio which is characteristic of the response of the animal with normally located adrenals to thoracic caval constriction. The transplanted adrenal gland responded to acute hemorrhage with marked increases in secretion rates of aldosterone, corticosterone and Porter-Silber chromogens. The present data demonstrate that neither intact renal and adrenal nerves nor increased renal venous pressure are essential to the hypersecretion of aldosterone and virtually complete sodium retention which follow thoracic inferior vena caval constriction. They further indicate that increased ACTH release is an important part of the response of the animal to acute hemorrhage.

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STUDIES ON THE DIFFERENCES IN ACTIVITY OF LUNG AND BRAIN THROMBOPLASTIN

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Recent reports have suggested that thromboplastin (Tpl) prepared from lung may reflect more accurately than does brain Tpl the changes produced in the prothrombin complex by coumarin derivatives (1, 2). As evidence for this, it has been observed that as factor VII concentration decreases in the plasma of Dicumarol[1]-treated patients, the prothrombin time obtained with lung Tpl becomes disproportionately longer than that obtained with brain Tpl (1). The inference from this observation has been that brain Tpl contains a factor VII-like activity, not possessed by lung Tpl. This activity would mask a lowered plasma concentration of factor VII and would result in spuriously short prothrombin times. Verstraete, Clark and Wright (1, 2) concluded that brain thromboplastins possess, although to a lesser degree, the disadvantage previously observed for Russell's viper venom (3, 4). The latter, by virtue of its intrinsic convertin-like activity, is insensitive to changes in factor VII concentration and is, therefore, no longer used in following anticoagulated patients.

Other observations, however, suggest that brain Tpl does not contain more factor VII activity than does lung and may, in fact, contain less. Mann and Hurn (5) have noted that contamination of the Tpl with small quantities of blood may result in marked enhancement of activity when tested on the plasma of patients receiving coumarin derivatives. If the factor VII-like activity of tissue Tpl was due to contamination by blood and not to intrinsic properties of Tpl itself, one would expect brain Tpl to show less such activity than lung Tpl, inasmuch as lung preparations are more heavily contaminated by blood during their preparation. In addition, Verstraete and associates (1) found that when Tpl solutions were adsorbed with $BaSO_4$, lung Tpl showed a pronounced decrease in activity when tested on the plasma of patients receiving Dicumarol, whereas brain Tpl showed virtually no change.

An alternative explanation for the longer prothrombin times obtained with lung Tpl is suggested by the observations of Conley, Morse and Stewart (6). They showed that, if plasma was diluted with saline, the prothrombin times obtained with lung Tpl became shorter than those obtained with the corresponding dilutions in $BaSO_4$-adsorbed plasma. This would suggest the presence in plasma of an inhibitor of lung Tpl.

The purpose of the present investigation was to study further the differences in activity of lung and brain Tpl.

MATERIALS AND METHODS

Collection of plasma. Nine parts of blood were mixed with one part of 0.1 M sodium oxalate and the plasma separated after centrifuging at 3,000 rpm for 10 minutes. Human plasma was used for all experiments. Normal plasmas were pooled by mixing equal parts from 5 donors.

$BaSO_4$-adsorbed plasma. Plasma was incubated with $BaSO_4$ (100 mg per ml of plasma) at 37° C for 10 minutes and then removed after centrifuging.

Commercial thromboplastins. Alban Permaplastin (AP) was used as the source of rabbit brain extract Tpl, and Difco Aplastin (DA) as the source of rabbit lung Tpl. The latter, a dry material, was reconstituted each day by extracting 100 mg of the powder with 4 ml of saline at 50° C for 10 minutes. The extracts were not frozen, as this resulted in considerable loss of activity.

Prepared thromboplastins. Brain and lung Tpl's were prepared from normal rabbits and from Dicumarol-poisoned rabbits. Brain Tpl's were prepared by acetone dehydration (7) and were stored in air-evacuated glass ampuls. Aliquots were reconstituted on the day of use by extracting 100 mg with 4 ml of normal saline at 50° C for 10 minutes. Lung Tpl was prepared as described by Johnson and Seegers (8). The first 24-hour saline extract of the ground lung was discarded. The second 48-hour extract was used as the source of thromboplastin. Rabbits that received Dicumarol were given

[1] Bishydroxycoumarin.

5 mg per kg of body weight each day for 4 days. When sacrificed, their prothrombin times (Permaplastin) were 2 minutes or longer.

Plasma prothrombin conversion inhibitor. To 50 ml of $BaSO_4$-adsorbed plasma was added an equal volume of saturated ammonium sulfate solution. After standing at 4° C for 10 hours, the material was centrifuged and the supernatant was removed. Saturated ammonium sulfate was then added to bring the final concentration to 80 per cent saturation. The precipitate which formed during 4 hours at 4° C was removed by filtration, dissolved in 5 ml of water and dialyzed until free of the sulfate.

"Albumin-free" plasma fraction. Equal volumes of $BaSO_4$-adsorbed plasma and saturated ammonium sulfate were mixed and allowed to stand 3 hours at 4° C. After centrifuging, the precipitate was washed twice with cold 50 per cent ammonium sulfate. It was dissolved in saline (one-half the original plasma volume) and dialyzed against normal saline until free of the sulfate.

Plasma eluate. Twenty ml of plasma was incubated with $BaSO_4$ as described. The $BaSO_4$ was washed with cold saline and eluted with 2 ml of 0.2 M sodium citrate.

Serum eluate. Blood was allowed to clot and after 6 hours at 37° C the serum was removed by centrifugation. This was adsorbed with $BaSO_4$ and citrate elution was carried out as for plasma.

Purified prothrombin. This preparation, containing Stuart factor but essentially free of factor VII activity, was kindly supplied by Dr. Sandor Shapiro of the National Institutes of Health.

One-stage prothrombin time (Quick). Equal parts of Tpl extract and 0.025 M $CaCl_2$ were mixed together and 0.2 ml of this mixture was added to 0.1 ml of plasma.

RESULTS

1. Differences in activity between brain and lung Tpl. Prothrombin times were determined using normal plasma diluted with saline and with $BaSO_4$-adsorbed plasma (Figure 1). At higher dilutions with $BaSO_4$ plasma, the lung Tpl gave increasingly longer prothrombin times than did brain Tpl, resulting in a wide divergence of the curves. When saline was used as a diluent, the difference between the two Tpl's was considerably less and the curves obtained were almost parallel. Moreover, with brain Tpl the dilution of plasma with saline resulted in considerably longer prothrombin times than did comparable dilution with $BaSO_4$-adsorbed plasma. When lung Tpl was used the curves obtained with both diluents were similar, and at high dilutions the values obtained with saline were even shorter than those with adsorbed plasma. These findings essentially confirm the observations of Conley and

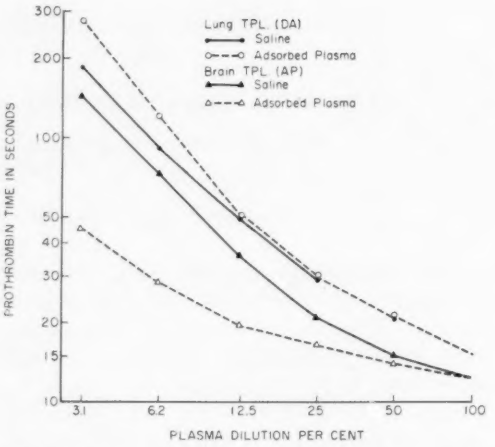

FIG. 1. THE EFFECT OF THE DILUENT ON THE PROTHROMBIN TIMES OBTAINED WITH BRAIN (AP) AND LUNG (DA) TPL. Pooled, normal plasma was diluted with normal saline and with $BaSO_4$-adsorbed plasma.

associates (6) and Verstraete and co-workers (1), and strongly suggest the presence in $BaSO_4$-adsorbed plasma of an inhibitor of lung Tpl that becomes more effective at increasing dilutions of plasma.

Further evidence for the presence of an inhibitor was obtained by diluting plasma with "albumin-free" plasma fraction. The curves obtained with

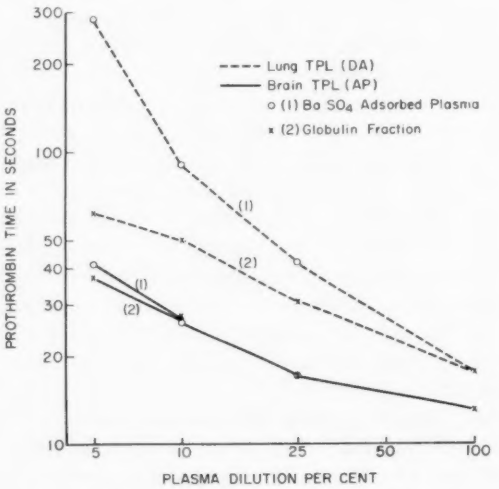

FIG. 2. PROTHROMBIN TIMES OF POOLED, NORMAL PLASMA DILUTED IN 1) $BaSO_4$-ADSORBED PLASMA, 2) "ALBUMIN-FREE" PLASMA FRACTION PREPARED FROM $BaSO_4$-ADSORBED PLASMA.

the two Tpl's were compared with those using the ordinary adsorbed plasma as diluent (Figure 2). When brain Tpl was used there was virtually no difference between the two diluents. With lung Tpl, however, the curve obtained with "albumin-free" plasma was much flatter and was now parallel to the curve obtained with brain Tpl.

2. Evidence that brain thromboplastin does not possess a greater factor VII-like activity than lung. It has been observed that Tpl obtained from the tissues of Dicumarol-poisoned animals is less active with the plasma of coumarin-treated patients than is Tpl from normal animals (9–12). According to Mann and Hurn (5), this difference reflects the contamination of tissue Tpl with blood. If the blood trapped in the tissues has been depleted of coagulation factors, the Tpl extracted from these tissues would be correspondingly less active. If brain Tpl contains more factor VII activity than lung, then Dicumarol poisoning would cause a greater change in the activity of brain Tpl. To test this, plasma was diluted with $BaSO_4$-adsorbed plasma and tested with brain and lung Tpl obtained from normal and Dicumarol-treated rabbits (Figure 3). The results show that pretreating animals with Dicumarol had a greater effect in decreasing the activity of lung Tpl, suggesting that

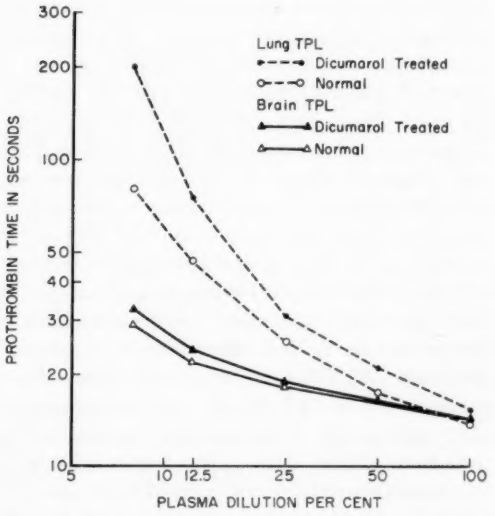

FIG. 3. PROTHROMBIN TIMES OF NORMAL PLASMA DILUTED WITH $BaSO_4$-ADSORBED PLASMA, USING BRAIN AND LUNG TPL FROM NORMAL AND DICUMAROL-TREATED RABBITS.

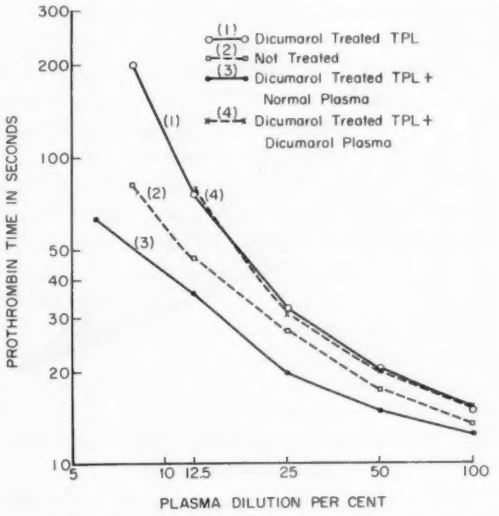

FIG. 4. PROTHROMBIN TIMES OF NORMAL PLASMA DILUTED WITH $BaSO_4$-ADSORBED PLASMA USING LUNG TPL 1) FROM RABBITS WHICH HAD RECEIVED DICUMAROL AND 2) FROM NORMAL RABBITS. Hemoglobin concentration of these extracts was 75 mg per 100 ml. Curve 3 shows the result of adding to the "Dicumarol-treated" Tpl 0.0025 ml of normal rabbit plasma per ml of Tpl solution, which corresponds roughly to a hemoglobin contamination of 50 mg per 100 ml. In curve 4 the same amount of plasma from a Dicumarol-treated rabbit was used.

factor VII activity is lower in brain than in lung Tpl.

The hemoglobin concentration in the brain Tpl solution was 1.0 mg per 100 ml, and in the lung Tpl, 80 mg per 100 ml. It was estimated that a hemoglobin concentration of 50 mg per 100 ml corresponded to a plasma contamination of 0.0025 ml per ml of thromboplastin extract. Therefore, this amount of plasma was added to the Dicumarol-treated lung thromboplastin and the effect on its activity was observed (Figure 4). The addition of normal rabbit plasma resulted in a markedly enhanced activity, while the addition of an equivalent amount of plasma from a Dicumarol-treated animal had no effect.

It is concluded that contamination of Tpl by blood alters the activity considerably and that, because of its greater contamination, lung is more affected than brain Tpl. The hemoglobin concentrations in the commercial Tpl's used throughout this study were 2 mg per 100 ml for brain, and 37 mg for lung Tpl.

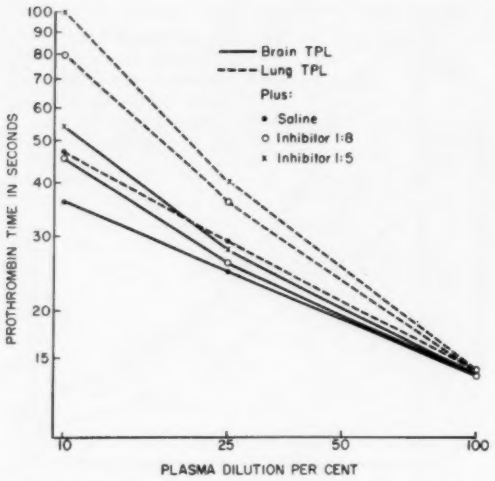

FIG. 5. EFFECT OF ADDING INHIBITOR TO NORMAL PLASMA DILUTED WITH SALINE: 0.2 ML TPL + 0.1 ML PLASMA + 0.02 ML OF NORMAL SALINE OR INHIBITOR.

3. Effect of plasma prothrombin-conversion inhibitor (PPCI) on brain and lung Tpl. PPCI diluted 1:5 and 1:8 was added to plasma diluted with saline and prothrombin times were performed. The results show that the prolongation of the prothrombin times at increasing dilutions of plasma was greater with lung than with brain Tpl (Figure 5).

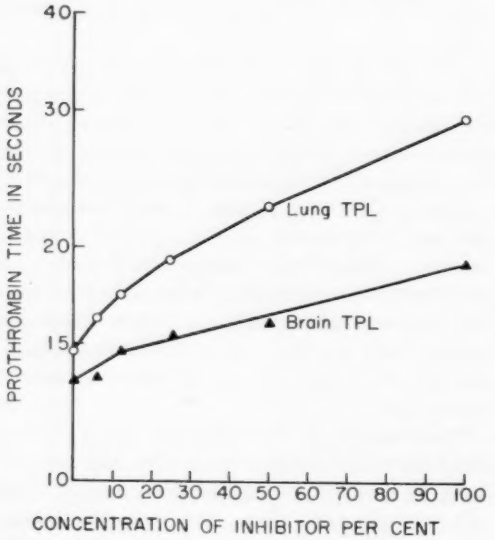

FIG. 6. EFFECT OF ADDING INHIBITOR TO NORMAL PLASMA: 0.2 ML TPL + CA + 0.1 ML PLASMA + 0.02 ML INHIBITOR.

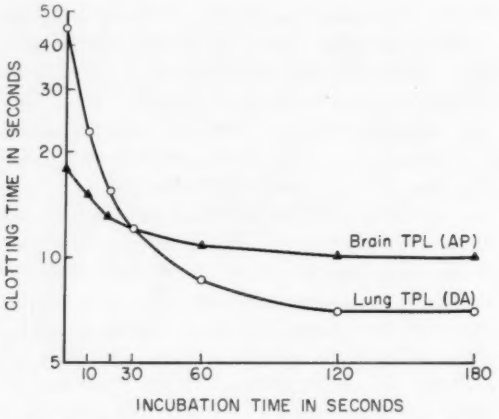

FIG. 7. EFFECT OF PRE-INCUBATING TPL + CA WITH PLASMA ELUATE BEFORE ADDING TO $BaSO_4$-ADSORBED PLASMA: 0.2 ML TPL + CA + 0.1 ML PLASMA ELUATE (1:20) INCUBATED FOR 2 MINUTES AT 37° C AND THEN 0.1 ML $BaSO_4$-ADSORBED PLASMA ADDED.

Increasing concentrations of PPCI were added to normal plasma. The effects on the prothrombin time are shown in Figure 6. Increasing the concentration of inhibitor has a greater effect in prolonging the prothrombin time with lung than with brain Tpl.

The activity of the two types of Tpl was further studied in inhibitor-free systems. Tpl + calcium mixture was pre-incubated for varying periods of time with plasma eluate, then added to $BaSO_4$-adsorbed plasma and the clotting time recorded (Figure 7). When plasma eluate diluted 1:20 was added to Tpl + Ca and the mixture was added immediately to adsorbed plasma without pre-incubation, lung Tpl gave a clotting time of 45 seconds, while brain gave 18 seconds. However, with pre-incubation, the clotting time obtained with lung Tpl sharply decreased. After 2 minutes of pre-incubation, it was shortened to 7 seconds, compared with 10 seconds for brain Tpl. When eluate dilutions of 1:40 were used, pre-incubation for 2 minutes with lung Tpl shortened the clotting time from 155 to 12 seconds, whereas with brain Tpl it was only shortened from 34 to 20 seconds. Thus, in the absence of inhibitor, lung Tpl appeared to be more active than brain Tpl.

The effect of inhibitor on the prothrombin conversion system was then studied. Plasma eluate

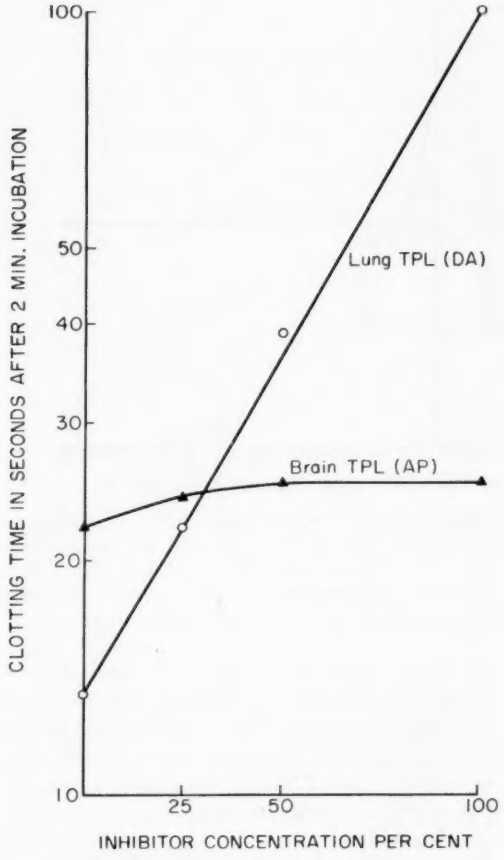

FIG. 8. EFFECT OF ADDING INHIBITOR DURING THE PRE-INCUBATION (2 MINUTES) OF TPL WITH PLASMA ELUATE: 0.2 ML TPL + CA + 0.1 ML PLASMA ELUATE (1:40) + 0.02 ML INHIBITOR DILUTED IN SALINE INCUBATED 2 MINUTES AT 37° C AND THEN 0.1 ML $BaSO_4$-ADSORBED PLASMA ADDED.

and Tpl + Ca were pre-incubated in the presence of increasing amounts of inhibitor for 2 minutes, at which time $BaSO_4$-adsorbed plasma was added. A dilution of plasma eluate of 1:40 was used in this experiment. Figure 8 shows the marked effect of the inhibitor on lung Tpl and the negligible effect on brain Tpl.

Studies were also performed with varying concentrations of eluate and inhibitor (Figure 9). Pre-incubation of Tpl + Ca + eluate was again carried out for 2 minutes in the presence of inhibitor. The results show that with no inhibitor, lung Tpl was more active (i.e., gave shorter prothrombin times) than did brain Tpl at all concentrations of plasma eluate. When inhibitor was added, there was virtually no effect on the curves obtained with brain Tpl, while the curves obtained with lung Tpl became widely divergent.

Further studies were then carried out to determine the reason for the greater activity of lung Tpl when pre-incubated with plasma eluate. During this pre-incubation, activation of at least two systems, factor VII and prothrombin (13–16), is known to occur.

To determine the effects of pre-incubation on the factor VII system, the Tpl + Ca mixtures were pre-incubated with a *serum* eluate for varying periods of time and then added to a mixture containing $BaSO_4$-adsorbed plasma and purified

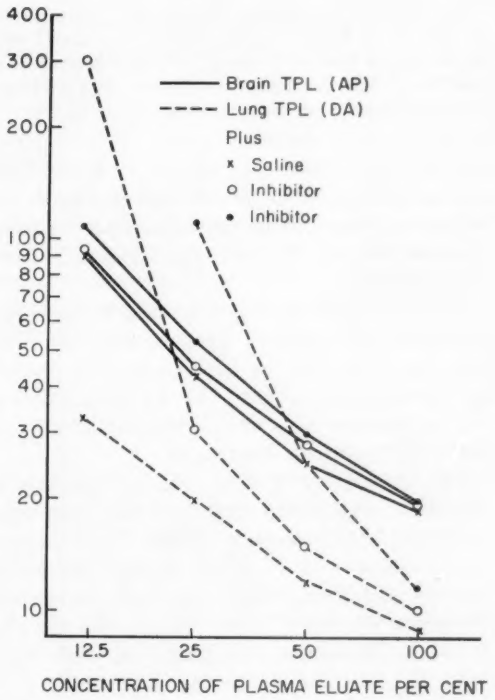

FIG. 9. EFFECT OF ADDING INHIBITOR DURING PRE-INCUBATION (2 MINUTES) OF TPL WITH PLASMA ELUATE: 0.2 ML TPL + CA + 0.1 ML PLASMA ELUATE OF VARYING CONCENTRATION + 0.02 ML NORMAL SALINE OR INHIBITOR INCUBATED 2 MINUTES AT 37° C AND THEN 0.1 ML $BaSO_4$-ADSORBED PLASMA ADDED. A 1:20 eluate dilution is represented as 100 per cent. Inhibitor 1:20 is represented by solid circles. Inhibitor 1:40 is represented by open circles.

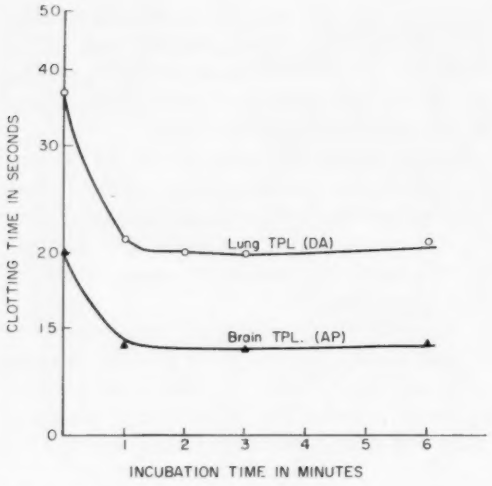

FIG. 10. EFFECT OF PRE-INCUBATING TPL WITH SERUM ELUATE: 0.2 ML TPL + CA + 0.1 ML SERUM ELUATE (1:40) INCUBATED FOR VARYING TIME AT 37° C FOLLOWED BY THE ADDITION OF 0.1 ML $BaSO_4$-ADSORBED PLASMA + 0.1 ML PURIFIED PROTHROMBIN (12.5 U ADDED).

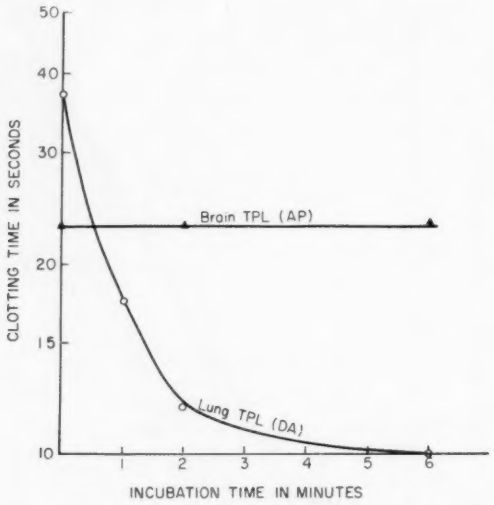

FIG. 11. EFFECT OF PRE-INCUBATING TPL WITH PURIFIED PROTHROMBIN: 0.2 ML TPL + CA + 0.1 ML PROTHROMBIN (12.5 U) INCUBATED FOR VARYING TIME AT 37° C, FOLLOWED BY THE ADDITION OF 0.1 ML $BaSO_4$-ADSORBED PLASMA + 0.1 ML SERUM ELUATE (1:40).

prothrombin. This is, in essence, a factor VII generation test and, as may be seen in Figure 10, the rate of change of activity is the same for both types of Tpl, and the brain Tpl develops greater maximal activity.

When, however, the Tpl + Ca mixtures are pre-incubated with purified prothrombin and then added to a mixture of $BaSO_4$-adsorbed plasma and serum eluate (Figure 11), the lung shows a marked increase in activity while the activity of brain Tpl is virtually unchanged.

The increase in activity of lung Tpl during pre-incubation with prothrombin probably represents actual thrombin formation. When Tpl + Ca was pre-incubated with 25 U of prothrombin for 3 minutes and then added to a $BaSO_4$-adsorbed fibrinogen solution (0.4 per cent), a clot was obtained in 50 seconds with lung Tpl, while with brain Tpl the clotting time was 12 minutes.

The possibility of contamination of lung Tpl with factor V was studied. The markedly greater thrombin-forming capacity of lung Tpl in the presence of purified prothrombin indicated that the lung Tpl probably contained a significant amount of factor V activity. To compare this activity with that in brain Tpl, prothrombin times were performed on plasma whose factor V concentration had been reduced by dilution with aged, normal plasma (Figure 12). With lung Tpl the curve was flatter, and at very low concentrations of factor V the prothrombin times were shorter than with brain Tpl. Freezing and thawing can reduce factor V activity of plasma. The reduction of Tpl activity of lung preparations after freezing may be a result of the destruction of contaminant factor V.

The possible mechanism of action of the inhibitor was next studied (Table I). When lung Tpl + Ca + serum eluate, 1:40, + prothrombin were

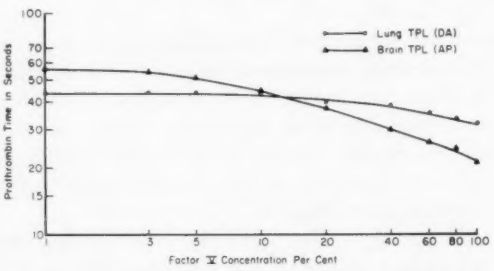

FIG. 12. EFFECT OF FACTOR V DILUTION ON PROTHROMBIN TIMES. Normal plasma was diluted with saline (1 to 100 per cent) and 0.1 ml was added to 0.9 ml of aged plasma. Prothrombin times were done on 0.1 ml of this mixture.

TABLE I

Action of inhibitors *

Experiment no.	Time added (min) 0	2	4	6	Clotting time (sec)
1	Ba† + Proth.‡				55
2		Ba + Proth.			21
3			Ba + Proth.		24
4		Proth.	Ba		10
5		Proth. + In.§			62
6		Proth.	In.	Ba	12
7		Proth.		Ba	13

* Two-tenths ml lung Tpl (DA) + Ca were added to 0.1 ml of serum eluate, 1:40, at zero time. The substances indicated were added at 0, 2, 4 and 6 minutes. Clotting times were obtained after the addition of $BaSO_4$-adsorbed plasma.
† $BaSO_4$-adsorbed plasma, 0.1 ml.
‡ Purified prothrombin (12.5 U).
§ Inhibitor, 0.02 ml.

mixed with $BaSO_4$ plasma without any pre-incubation, a clotting time of 55 seconds was obtained. If the Tpl + Ca was pre-incubated with serum eluate *before* adding the adsorbed plasma and prothrombin (thereby activating factor VII), a maximal shortening of the clotting time (21 seconds) was achieved after 2 minutes of pre-incubation (Experiments 1, 2 and 3).

Using this procedure, the experiment was conducted as follows: Tpl + Ca + serum eluate were incubated for 2 minutes, then prothrombin was added and after 2 minutes more the adsorbed plasma was added; clotting occurred at 10 seconds. If, in this sequence, inhibitor was added at the same time as the prothrombin, clotting occurred at 62 seconds.

If the addition of inhibitor was delayed until thrombin had formed, there was no inhibition. Thus, if prothrombin was added at 2 minutes, inhibitor at 4 minutes, and adsorbed plasma at 6 minutes, clotting occurred at 12 seconds. It would seem that the inhibitor acts by interfering with the conversion of prothrombin to thrombin and is effective even after factor VII has been activated.

4. Studies of lung and brain Tpl on patients receiving coumarin drugs. Prothrombin times were performed on an unselected group of patients who were receiving Coumadin,[2] using lung and brain Tpl. The prothrombin activity was calculated as percentage of normal plasmas diluted with adsorbed plasma and with saline (Figure 13). In all cases the prothrombin times obtained with lung Tpl were longer than those obtained with brain Tpl. When prothrombin activity was calculated from the saline dilution curves, the values were always lower for lung than for brain Tpl. However, on the adsorbed plasma dilution curve, the value for lung Tpl was higher. Similar results were obtained on a patient who was followed throughout a course of therapy with Coumadin (Figure 14).

The choice of diluent markedly affected the calculation of percentage activity when brain Tpl was used; with saline, the values were considerably higher than with adsorbed plasma. With lung Tpl, the calculated activity was essentially the same for both diluents.

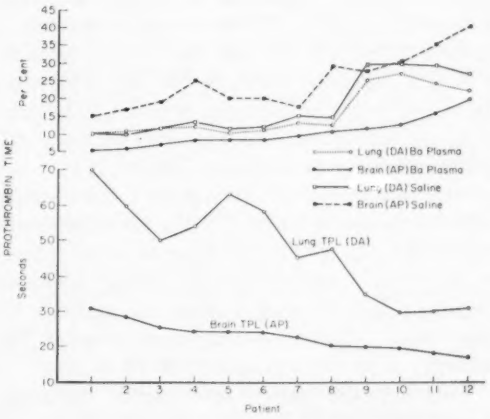

FIG. 13. PROTHROMBIN DETERMINATIONS ON 12 PATIENTS RECEIVING COUMADIN, USING LUNG AND BRAIN TPL. The results are expressed in seconds and percentage of normal activity, using reference dilution curves obtained with $BaSO_4$-adsorbed plasma or saline.

[2] Warfarin sodium.

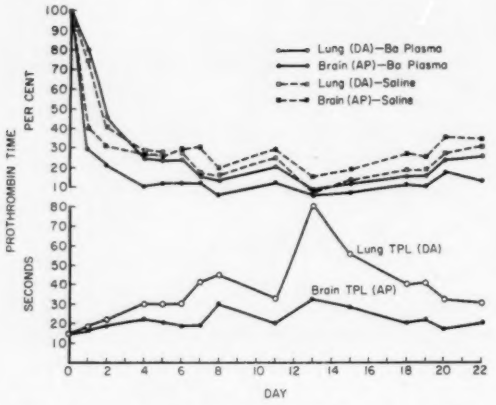

FIG. 14. PROTHROMBIN DETERMINATIONS ON A SINGLE PATIENT WHO RECEIVED COUMADIN, USING LUNG AND BRAIN TPL. Calculation of percentage was obtained from both saline and $BaSO_4$-adsorbed plasma dilution curves.

DISCUSSION

When different tissue Tpl's are used to perform prothrombin times on the plasma of a patient receiving coumarin derivatives, widely varying results may be obtained (1, 2, 6, 17–21). In particular, it has been noted that lung Tpl's give longer prothrombin times than do brain Tpl's. Verstraete and co-workers also showed that the disproportionately long prothrombin times obtained with lung Tpl and plasma diluted with $BaSO_4$-adsorbed plasma was due to a dilution not of prothrombin, but of factor VII (1). They concluded that this difference might represent a greater factor VII-like activity of brain extracts, and they suggested therefore that lung Tpl might be a more sensitive indicator of factor VII depletion. Other authors have also stressed the possibility of "activators" in tissue Tpl (19, 20). Since a reduction in factor VII appears to be a major effect of coumarin drugs (22, 23), the employment of a tissue Tpl sensitive to changes in its activity is obviously important for controlling coumarin therapy. It is for such reasons that Russell's viper venom, which apparently contains factor VII activity, is not recommended (3, 4).

However, the longer prothrombin times obtained with lung extracts do not necessarily mean that brain Tpl contains more factor VII-like activity. The previous work of Conley and associates (6) suggested that lung Tpl might be more sensitive to the effect of an inhibitor in $BaSO_4$-adsorbed plasma. In view of the findings of Verstraete and co-workers (1, 2) this inhibitor might involve the factor VII system. Such plasma inhibitors of the prothrombin conversion system have been described. Wagner, Brannan and Brinkhous (24) described an antagonist of factor VII present in the fraction of canine plasma which is precipitated by ammonium sulfate between 70 and 80 per cent saturation. Schaefer, Therriault and Jensen (25) described an inhibitor in rabbit serum which precipitated between 50 and 80 per cent saturation and which inhibited the interaction of prothrombin-converting factor (presumably factor VII) and prothrombin. In addition, a lipid-extractable inhibitor in plasma with the properties of an antithromboplastin has been described (26), which may act by inhibiting the activation of factor VII (25). Other Tpl inhibitors have been reported by Overman (27) and by Lanchantin and Ware (28).

Our studies indicate that an inhibitor, precipitated by ammonium sulfate between 50 and 80 per cent saturation, is present in human plasma. This inhibitor is more active when lung extract is used as a source of tissue Tpl than when brain extract is used. The presence of such an inhibitor would explain why lung Tpl gives longer prothrombin times at increasing dilutions of plasma in $BaSO_4$-adsorbed plasma than does brain Tpl. It would also explain the shortening of the prothrombin time observed for lung Tpl when saline instead of $BaSO_4$-adsorbed plasma was used as a diluent. Therefore, the shorter prothrombin times obtained with brain Tpl on the plasma of anticoagulated patients need not mean that brain extract contains more factor VII activity, nor is it necessarily a less sensitive Tpl.

The importance of expressing prothrombin activities as percentage of normal rather than as absolute prothrombin times has been stressed (17). In this respect, the choice of diluent used in obtaining a normal curve is important. The ideal diluent would be human plasma which has been depleted of factors reduced by coumarin derivatives. Conley and associates (6) showed that $BaSO_4$-adsorbed plasma best approximates this. Our studies have shown that, when this diluent is used, brain Tpl, although giving a shorter prothrombin time, actually reveals a lower percentage of activity than does lung Tpl.

SUMMARY

The differences in activity between lung and brain thromboplastin (Tpl) have been studied—in particular, the greater prolongation of the prothrombin times obtained with lung Tpl at decreasing factor VII concentration. It was found that lung extract was actually more active when the prothrombin conversion system was studied in the absence of plasma inhibitors. In the presence of purified prothrombin, lung Tpl produced a greater coagulant effect than did brain thromboplastin.

The values obtained with lung Tpl are evidently subject to two uncertain variables: *1*) the amount of blood remaining in the tissue extract, and *2*) the susceptibility of lung Tpl to the effect of an inhibitor present in normal plasma. Thus, one variable tends to speed up the prothrombin time; the other tends to slow it down. The results obtained with lung Tpl could vary with factors other than the "prothrombin complex" in the plasma of the patient.

Brain Tpl is not subject to these variables. It contains insignificant amounts of blood and it is not affected by the inhibitor.

The practice of mixing lung and brain Tpl in unspecified proportions, as recommended by the USP (16th ed.), would seem to compound the variables and the problems of controlling anticoagulant therapy.

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THE EFFECT OF INDUCED HYPERAMMONEMIA ON RENAL AMMONIA METABOLISM * †

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Although the excretion of ammonia into urine has been extensively studied, there is little information in animals or man concerning the quantitative and regulatory aspects of the ammonia released into the renal veins. Previous observations in this laboratory and in others have demonstrated that the kidney consistently releases ammonia into the systemic circulation of normal subjects and patients with liver disease whose arterial ammonia concentrations are normal (1–5). Patients with liver disease and moderate to marked hyperammonemia, however, usually release minimal quantities of ammonia into their renal veins and occasionally exhibit renal uptake of ammonia from the circulation (2). In order to further define the possible role of the blood ammonia concentration on renal ammonia release, acute hyperammonemia has been induced in normal subjects and the subsequent changes in renal vein ammonia release and urine ammonia excretion determined.

METHODS

Nine patients without hepatic or renal disease were studied. All were hospitalized ambulatory males ranging in age from 28 to 49 years.

Subjects were studied in the recumbent position after an overnight fast. In order to initiate a water diuresis, 1,000 to 1,500 ml of water was ingested 30 minutes to 1 hour before each procedure. A constant intravenous infusion (Bowman pump) which delivered 14 to 18 mg of para-aminohippurate (PAH) per minute was maintained throughout the study period and was preceded by a priming dose calculated to provide plasma levels of approximately 2 mg per 100 ml. The water diuresis was maintained throughout the procedure by the intravenous infusion of 5 per cent dextrose and water at a rate of 15 to 20 ml per minute. Arterial samples were obtained from a brachial artery and renal venous samples from the right renal vein by way of a no. 8 catheter. Control voided urine specimens were collected during and after equilibration of the PAH infusion. Baseline arterial and renal venous samples were begun 45 to 60 minutes after the PAH infusion was started. Each patient received an intravenous infusion of 0.155 M ammonium lactate at a rate of 0.50 mEq of ammonia per minute for 30 minutes and 0.75 mEq of ammonia per minute for the subsequent 15 minutes. Serial arterial-renal venous ammonia differences were obtained at 15-minute intervals during the infusion and at the 15 minute post-infusion period. Voided urine samples collected in an oil-toluene mixture were obtained at 15- to 30-minute intervals during the infusion and in 4 subjects for 30 to 60 minutes after the infusion was terminated.

Blood and urine ammonia was measured by a modification (6) of the microdiffusion method of Brown, Duda, Korkes and Handler (7). PAH concentration in blood and infusion media was determined by the method of Selkurt (8), with the N-napthyl ethylenediamine dihydrochloride recrystallized with hydrochloric acid as described by Bratton and Marshall (9). The pH of whole blood and urine was measured with a Cambridge model R pH meter equipped with an enclosed glass electrode. Measurements were made at room temperature usually at 25° to 26° C and the results corrected to 37° C by Rosenthal's factor (10). The CO_2 content of whole blood was determined by the method of Van Slyke and Neill (11). The CO_2 tension was calculated from this value, the pH and the hemoglobin concentration by the line chart of Van Slyke and Sendroy (12), using a pK of 6.11. Oxygen content and saturation were determined by the spectrophotometric method of Hickam and Frayser (13). Hematocrits were determined in duplicate from arterial blood in Wintrobe tubes centrifuged at 3,000 rpm for 30 minutes. Renal plasma flow (RPF) was calculated by a modification (14) of the Fick principle using the formula $RPF = IR/(A\text{-}R)$, where IR represents the infusion rate of PAH in milligrams per minute and A-R represents the PAH concentration in arterial and renal venous blood. The PAH concentration in arterial blood remained essentially constant throughout each study period. Renal blood flow was obtained using RPF and hematocrit. Details of this method and its comparison with standard clearance techniques have been described previously (3). Release or uptake of ammonia by the kidney was calculated from the arterial-renal venous ammonia differences and renal blood flow. Renal oxygen consumption was determined using arterial-renal venous oxygen differences.

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TABLE I

The effect of induced hyperammonemia on release of ammonia by the kidney

Patient	Time	Arterial NH_3	Renal NH_3 A-V	Renal O_2 A-V	Renal blood flow	Release of NH_3 into renal veins	Removal of NH_3 from circulation	Renal O_2 consumption	Arterial pH	Arterial Serum CO_2	Arterial pCO_2
	min	*μg/100 ml*		*vol %*	*ml/min**	*μEq/min*		*ml/min*		*vol %*	*mm Hg*
	Rest †	88	−36	1.18	2,220	47		26.2	7.37	48.6	38.1
W.H.	15	292	+67		2,240		88				
	30‡	283	+24	1.13	2,443		35	27.6	7.34	49.6	41.5
	45	425	+29		2,426		72				
	60§	108	−25	1.07	2,445	36		26.2	7.35	49.5	40.2
	Rest †	73	−37	0.92	1,023	23		9.4	7.34	53.0	43.8
J.H.	15	249	+22		1,144		15				
	30‡	281	+39	1.02	1,223		28	12.5	7.34	53.5	44.7
	45	428	+50		1,191		35				
	60§	124	−20	1.01	1,310	16		13.2	7.33	53.9	45.2
	Rest †	97	−19	1.08	1,622	18		16.3	7.38	47.1	35.6
E.Q.	15	319	+72		2,002		85				
	30‡	311	+80	0.72	1,743		82	12.5	7.37	47.6	36.7
	45	540	+132								
	Rest †	95	−48	0.59	2,727	77		14.1	7.35	63.5	51.1
R.L.	15	206	−24		2,500	36					
	30‡	223	−28	0.69	2,345	39		16.2	7.36	62.7	49.5
	45	348	+ 4		2,400		6				
	60§	125	−65	0.98	2,500	91		24.5	7.36	63.9	51.1
	Rest †	55	−67	0.05					7.32	54.8	47.8
C.L.	15	235	−39								
	30‡	213	−58	0.18					7.34	53.6	45.1
	45	540	−34								
	60§	98	−64	0.28					7.34	53.4	44.9
	Rest †	88	−35	0.78	1,641	34		12.8	7.33	53.2	45.5
I.W.	15	238	+28		1,995		35				
	30‡	290	+53	0.68	2,219		69	15.1	7.34	51.9	43.5
	45	346	+87								
	60§	100	− 9	0.94					7.38	51.9	39.5
	Rest †	79	−21	0.71	930	12		6.8	7.38	57.0	43.0
E.H.	15	212	+12		1,066		8				
	30‡	249	+ 5	0.91	1,070		3	7.7	7.36	57.0	45.0
	45	397	+53		1,085		34				
	60§	102	−24	1.13	1,141	15		12.8	7.38	56.4	42.9
	Rest †	70	−54	1.18					7.37	54.4	41.6
R.S.	15	250	+34								
	30‡	236	+45	1.68					7.36	55.1	41.8
	45	459	+111								
	60§	94	−36	1.69					7.38	56.1	43.8
	Rest †	79	−35	0.86	1,204	25		10.4	7.34	53.2	44.5
E.G.	15	332	+62		1,124		41				
	30‡	234	−35	1.04	1,181	25		12.3	7.30	54.6	49.3
	45	330	+27		1,015		16				
	60§	92	−26	0.79	1,358	21		10.5	7.31	54.7	48.2

* Instantaneous values obtained at indicated time periods.
† Ammonium lactate infused at 0.5 mEq of NH_3 per minute from 0 to 30 minutes.
‡ Ammonium lactate infused at 0.75 mEq of NH_3 per minute from 30 minutes to 45 minutes.
§ 15 minute post-ammonium lactate infusion.

RESULTS

The data obtained from blood analyses in each subject as well as mean values before, during and after intravenous ammonium lactate are presented in Tables I and II. An abrupt increase in arterial ammonia concentration occurred in each subject. Mean levels were 179, 180 and 344 μg per 100 ml higher than baseline values at the 15, 30 and 45 minute infusion periods, respectively. Fifteen minutes after the infusion was terminated, the mean arterial concentration was 105 μg per 100 ml (mean control arterial ammonia concentration, 80 μg per 100 ml). No mental or neurologic abnormalities were observed during the hyperammonemic period, nor were there significant changes observed in arterial pH or calculated pCO_2.

Negative baseline arterial-renal venous ammonia differences, indicating ammonia release and averaging 39 μg per 100 ml, were promptly altered by the ammonium lactate infusion. In seven subjects these alterations were quantitated using renal blood flows. During the control period, a mean of 34 μEq of ammonia per minute was released into the renal venous circulation. At the 15 minute period of infusion, an average of 34 μEq of ammonia per minute was removed from the circulation by the kidneys. Removal tended to decrease somewhat at the 30 minute period but increased again during the peak arterial ammonia concentration. Fifteen minutes after the infusion was discontinued, release of ammonia into the renal veins was again evident and approximated values obtained during the control periods.

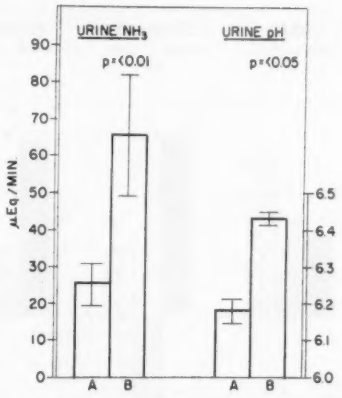

FIG. 1. URINE AMMONIA EXCRETION AND pH DURING AMMONIUM LACTATE-INDUCED HYPERAMMONEMIA (MEAN VALUES OF EIGHT SUBJECTS ± 1 SD). **A** represents control values and **B** represents hyperammonemic values.

Mean urine ammonia excretion and pH in four catheterized subjects and in four additional individuals infused with similar quantities of ammonium lactate are shown in Figure 1. Urine ammonia excretion increased significantly from a control of 25 to 65 μEq per minute ($p < 0.01$) during the hyperammonemic period. This augmented urine ammonia excretion occurred during the first 15 to 20 minutes of hyperammonemia and remained two to three times higher than baseline for 20 to 40 minutes after the infusion was dis-

TABLE II

Comparison of mean values for renal ammonia release after intravenous infusion of ammonium lactate

Time		Arterial NH_3	Renal NH_3 A-V	Renal O_2 A-V	Renal blood flow	Release of NH_3 into renal veins	Removal of NH_3 from circulation	Renal O_2 consumption	Arterial pH	Arterial Serum CO_2	Arterial pCO_2
min		*µg/100 ml*		*vol %*	*ml/min*	*µEq/min*		*ml/min*		*vol %*	*mm Hg*
Rest	$\bar{d}$*	80.4	−39	0.82	1,623	34		13.7	7.35	53.9	43.4
†											
15	$\bar{d}$	259	+26		1,724		34				
	p‡	<0.01	<0.01		<0.01		<0.01				
30§	$\bar{d}$	258	+14	0.89	1,746		22	14.8	7.35	54.0	44.1
	p	<0.01	<0.01	>0.1	>0.1		<0.01	>0.1	>0.1	>0.1	>0.1
45	$\bar{d}$	424	+51		1,623		33				
	p	<0.01	<0.01		>0.1		<0.01				
60‖	$\bar{d}$	105	−34	0.86	1,751	37		17.4	7.35	55.0	44.5
	p	<0.01	>0.1	>0.1	<0.01	>0.1		>0.1	>0.1	>0.1	>0.1

* Mean values at the time periods indicated.
† Ammonium lactate infused at 0.5 mEq of NH_3 per minute from 0 to 30 minutes.
‡ Significance as compared with control values.
§ Ammonium lactate infused at 0.75 mEq of NH_3 per minute from 30 to 45 minutes.
‖ 15 minute post-ammonium lactate infusion.

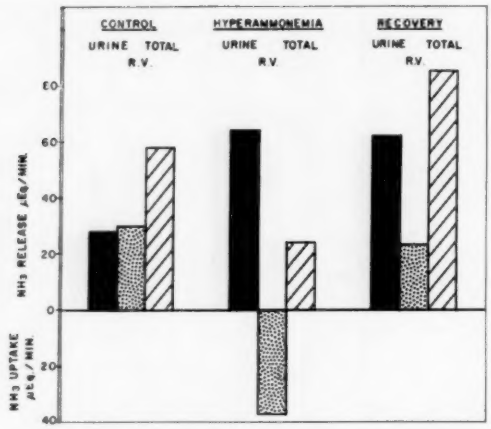

FIG. 2. RENAL AMMONIA BALANCE BEFORE, DURING, AND AFTER AMMONIUM LACTATE-INDUCED HYPERAMMONEMIA (MEAN VALUES OF FOUR SUBJECTS). R.V. represents net ammonia balance across the renal circulation.

continued in the four subjects who had continuous collections. Accompanying this increased urine ammonia excretion was a slight but significant increase in mean urine pH, 6.18 to 6.43. Urine flow decreased slightly in six of the eight subjects during the study (mean control urine flow = 10.8 ml per minute, mean infusion urine flow = 7.1 ml per minute).

Simultaneous observations obtained in four subjects permit calculations of net renal ammonia balance. Mean values are presented in Figure 2. During the control period, total bidirectional ammonia release was 56 μEq per minute. The quantity released into the renal veins averaged 52 per cent of the total. Although urine ammonia excretion increased significantly in this group ($p < 0.02$) during the hyperammonemic period, this was partially balanced by the renal uptake of ammonia from the circulation (mean uptake = 37 μEq per minute). During the recovery period, release of ammonia into the renal veins was again observed. Urine ammonia excretion remained at levels similar to those of the infusion period and mean total release was slightly but not significantly greater than that during the control period.

DISCUSSION

The concentration of ammonia in arterial blood is the resultant of a number of factors, including the rate of production and utilization by various tissues and the rate of diffusion into and out of cells. In normal subjects and patients with liver disease without hyperammonemia, the blood ammonia appears to be largely of renal origin (1, 2). Liver (2, 4), muscle (15, 16), and brain (15, 17), as measured by A-V differences, primarily serve to remove ammonia from the circulation under basal conditions. The present study provides quantitative data on the amount of ammonia normally released into the renal venous circulation and also indicates that the kidney's role as a donor of ammonia to the circulation can be abruptly altered by acutely increasing the blood ammonia concentration.

Recent quantitative studies in cirrhotics with chronic hyperammonemia have shown a marked decrease in renal vein ammonia release when compared to subjects with normal blood ammonia concentrations (18). Indeed, net removal of ammonia from the circulation has occasionally been observed (2). The notion that the level of the arterial ammonia concentration may be a factor governing the release of ammonia into the renal veins is supported by the results presented in the present acute experiments. In each subject, hyperammonemia induced by intravenous ammonium lactate was associated with a prompt decrease in renal vein ammonia release, with net uptake of ammonia from the circulation by the kidney being observed in eight of nine studies. Further evidence to support the role of the arterial ammonia concentration may be obtained from the prompt return to resting release within 15 minutes after ammonium lactate was discontinued, a time when the arterial ammonia concentrations were normal to slightly elevated. The precise level of arterial ammonia concentration at which uptake occurs is not evident from these data. It is apparent, however, that this level is significantly above normal and that there is considerable individual variation. Subject C.L., for example, continued to release ammonia despite an arterial concentration of 540 μg per 100 ml, whereas J.H. exhibited uptake at an arterial concentration of 240 μg per 100 ml.

The precise mechanism involved in the movement of ammonia from tubular cell to tubular urine and peritubular fluid has not been clearly elucidated. Currently available evidence based primarily on urine studies suggests that ammonia

escapes from the renal tubular cells as molecular NH_3 by a process of passive diffusion (19–21). The relative amounts appearing in each fluid, i.e., tubular urine and peritubular fluid, appear to depend on intracellular ammonia concentration, the pH of each surrounding fluid, and the fluid flows past the cell membranes. Under normal conditions, the escaping NH_3 rapidly traps protons forming ammonium ion (NH_4^+). Since cell membranes in general are relatively impermeable to NH_4^+, back diffusion into the tubular cells is minimized. Ammonia in blood even during extreme hyperammonemia exists primarily in the NH_4^+ form. The concentration of NH_3 represents only a minute fraction of the total blood ammonia concentration (22), and in the present study is obviously of insufficient magnitude to explain the alterations in renal ammonia balance across the renal circulation during ammonium lactate infusion. It would seem likely, therefore, that the renal tubular cells removed significant quantities of NH_4^+ from the blood during these experiments. Liver, muscle and brain also appear to remove NH_4^+ under similar conditions of induced hyperammonemia (17, 18).

The level of circulating ammonia also appears to affect the quantity of ammonia excreted into urine. A significant increase in mean urine ammonia excretion occurred in association with mean net removal of ammonia from the circulation. An increase in tubular ammonia secretion probably occurred during this period since only 40 to 50 per cent of the observed increment can be explained by filtration, even assuming no reabsorption during tubular passage. The augmented urine ammonia excretion occurred in association with a slight but significant increase in mean urine pH. Under most conditions, acute increases in ammonia excretion are associated with decreases in urine pH since increased H^+ ion concentrations facilitate the escape of ammonia from the tubular cells. The observed increase in urine pH could result from an increased exchange of cellular NH_4^+ for urinary sodium (23), or from increased diffusion of NH_3 from cell to urine, thereby trapping H^+ ions and thus allowing further H^+ ions for sodium exchange. A similar pattern of augmented urine ammonia excretion in association with an increase in urine pH has been observed following amino acid loads (24, 25). Presumably the common factor in each of these situations is an increase in renal tubular cell ammonia concentration.

Although there are relatively few observations on the relationship of resting urine pH to the release of ammonia into the renal veins, studies previously reported (3) suggest that there is little correlation within a range of urine pH of 4.99 to 7.15. It has been clearly shown, however, that acute increases in urine pH following carbonic anhydrase inhibition (3, 26) or metabolic alkalosis (18) are associated with rapid increases in ammonia released into renal veins, a rapid decrease in urine ammonia excretion, and an essentially unchanged total bidirectional release. These changes appear to occur irrespective of the level of control arterial ammonia concentration.

Quantitatively, the increase in urine ammonia excretion in these experiments was partially balanced by the renal uptake of ammonia from the circulation. Indeed, the magnitude of this uptake was such that it more than made up for the increase in urine ammonia excretion and resulted in a significant decrease in net release of ammonia by the kidney in the four patients who had simultaneous measurements. These observations suggest that net renal ammonia production was decreased during the ammonium lactate infusion.

The origin of urine and renal vein ammonia and the mechanisms of its escape from or entry into renal tubular cells are not answered by these experiments. Regardless of the mechanisms involved, it is clear that the kidney serves to lower the blood ammonia concentration during hyperammonemia by decreasing its release and by increasing urine ammonia excretion. These alterations would appear to be of clinical importance in patients with liver disease by aiding in the regulation of blood ammonia concentration. Since moderate amounts of oral ammonium chloride result in significant and often marked elevations of arterial ammonia concentration (27), the present observations may also be of importance in partially explaining the augmented urine ammonia excretion seen during chronic ammonium chloride loads (19, 28). The increased urine ammonia excretion which attends amino acid loads is also associated with striking elevations of the blood ammonia concentration (29). During intravenous glycine infusions, renal ammonia production ap-

pears to increase, and presumably the increased ammonia appearing in the renal veins partially accounts for the increased blood ammonia concentration (30). Whether the increase in urine ammonia excretion seen following oral or intravenous loading with other amino acids is related to augmented production or hyperammonemia must await more definitive studies.

SUMMARY

The release of ammonia into the renal veins was determined in nine normal subjects before, during and after the intravenous administration of isotonic ammonium lactate. Concomitant measurements of urine ammonia excretion were obtained in four catheterized subjects and in four additional individuals infused with similar quantities of ammonium lactate.

Hyperammonemia was associated with a prompt decrease in the amount of ammonia normally released into the renal veins with uptake of ammonia from the blood by the kidneys being observed in eight of nine subjects. Release reappeared and approximated control values 15 minutes after the infusion was terminated, a time when the blood ammonia concentrations were normal to slightly elevated.

Urine ammonia excretion increased two- to threefold during the infusion in association with a slight increase in urine pH, suggesting augmented tubular secretion. The resultant of these two processes, however, indicates an over-all decrease in bidirectional release and presumably in total ammonia production.

These observations provide additional evidence of the kidney's role in the regulation of the blood ammonia concentration and, in addition, demonstrate that the arterial ammonia concentration significantly influences urine ammonia excretion.

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LYMPHATIC FLOW IN HUMAN SUBJECTS AS INDICATED BY THE DISAPPEARANCE OF I^{131}-LABELED ALBUMIN FROM THE SUBCUTANEOUS TISSUE * †

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Prior studies in animals have indicated that the lymphatic system returns extravascular protein to the blood stream (1–8). Lewis injected horse serum subcutaneously into dogs and found that it appeared in the thoracic duct lymph before it was detected in the blood (4). Similar observations were made by Field and Drinker (5). Conklin, after injecting foreign proteins into the circulating blood of the frog, observed that they passed through the skin capillaries into the lymphatics (6). More recently Courtice, Simmonds and Steinbeck have shown that homologous serum protein labeled with Evans blue is absorbed by the lymphatics when introduced into the pleural or peritoneal cavities (7, 8).

In man, lymphatic function has been studied mainly by outlining the lymphatics visually with vital dyes injected into the tissues (9–11). The interpretation of these studies has been based primarily on the local distribution of the dyes in the tissues. The removal of particles and colloids by the lymphatics also has been studied (12–14). However, the results of these experiments have been influenced by the local reaction of the tissue to the injected foreign substance.

The present study was undertaken to determine whether the rate of disappearance of human serum albumin injected into the subcutaneous tissues might give some estimate of lymphatic function in man (15).

MATERIAL AND METHODS

Hospitalized patients with and without edema were studied. All patients had been at bed rest for 3 to 5 days without showing a variation in weight of more than 2 pounds or a measurable change in the size of the extremities. Throughout the study the patients remained at bed rest without significant change in body weight or in the circumference of the injected limb.

Fifteen patients in the control group had no history or physical findings suggesting cardiovascular or peripheral vascular disease. Their limbs were nonedematous and appeared normal.

Five patients had had lymphedema of an entire upper extremity for 3 to 15 weeks following radical mastectomy for carcinoma of the breast. One patient had had lymphedema of the lower extremity of 9 weeks' duration, resulting from a radical groin resection for malignant melanoma. In all 6 patients the edematous extremity pitted to local pressure. However, the venous pressure in the involved as well as in the opposite normal limb was in the normal range.

Seven patients had had edema, caused by peripheral venous obstruction, for 1 to 5 weeks. The edema of the involved extremities pitted to a marked degree. Venous obstruction was due to femoral or popliteal vein thrombosis in 4 patients, to compression of the inferior vena cava by an abdominal neoplasm in 2 other patients, and to narrowing of the superior vena cava by an inflammatory mediastinal lesion in another patient. In those cases of vena caval block, the venous pressure distally was over 350 mm of water.

Fifteen patients in severe congestive heart failure had enlargement of the heart, pulmonary congestion by X-ray, venous distention, marked pitting edema of the legs, elevated venous pressure and prolonged circulation time. The heart failure with peripheral edema had been present for 1 to 5 weeks and was being treated with salt restriction and maintenence of digitalis but without diuretics. Three patients with rheumatic heart disease, 2 with hypertensive heart disease and 1 with pulmonic stenosis were restudied after their edema had disappeared following further medical therapy with additional digitalis and frequent injections of mercurials. The hypertensive patients were also given antihypertensive drugs with return of their blood pressures close to normal

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levels. One other patient with constrictive pericarditis was restudied after pericardectomy at which time he was fully compensated, as revealed by the findings of right heart catheterization.

Six patients with Laennec's cirrhosis and 4 patients with nephrotic syndrome had peripheral edema associated with hypoproteinemia. The edema of the legs was of 2 to 7 weeks' duration and pitted markedly. The nephrotic syndrome was caused by amyloidosis in 2 patients, intercapillary glomerulosclerosis in 1 other patient, and glomerulonephritis in the remaining patient. All the patients had a normal venous pressure and were receiving a diet restricted in salt.

Twenty to 30 μc of a 1 per cent solution of I^{131}-labeled human serum albumin [1] was administered subcutaneously into an extremity in a volume of 0.03 to 0.05 ml using a 25 gage needle and a tuberculin syringe. In all studies on paired extremities and in repeat studies in cardiac patients the dose and volume of labeled albumin injected into the limbs was identical. The sites of injection were similarly controlled. To prevent leakage of the radioactive protein from the puncture wound, the labeled albumin was injected 30 to 35 mm from the site of puncture by advancing the needle subcutaneously. After the needle was withdrawn the puncture site was sealed with collodion. The site of injection was then externally monitored at intervals of 10 to 15 minutes for the first hour and every 2 to 3 hours for the next 12 hours. Thereafter, the site was monitored 4 times a day until the injected radioactivity had disappeared from the limb. The activity at the site of injection was corrected for "background" by subtracting the radioactivity of the uninjected limb from the activity of the injected limb. In certain cases, a scintogram scan of the entire limb was made and compared with that of the opposite control limb. The thyroid gland was externally monitored at intervals of 6 to 12 hours. Daily total urine collections were made and assayed for radioactivity. Plasma samples were drawn at times at which the limb was monitored. Total radioactivity, trichloroacetic acid (10 per cent) precipitable radioactivity, and the radioactivity in the supernatant were determined in the plasma samples. More than 96 per cent of the radioactivity was precipitable with the trichloroacetic acid, and all of the radioactivity was contained in the albumin fraction separated by electrophoresis. Radioactivity of plasma and urine samples was assayed in a well-type sodium iodide crystal. The measurements of radioactivity were related to appropriate standards.

In a number of subjects after the labeled albumin had completely disappeared from the tissue, 0.1 ml of a solution containing 10 to 15 μc of $Na^{24}Cl$ or of NaI^{131} was injected subcutaneously into an area which had previously been injected with I^{131}-labeled albumin. The rate of disappearance of these radioactive ions was determined by monitoring the injection site at intervals of 1 to 3 minutes. The activity at the site of injection was corrected for background (final plateau reached after clear-

[1] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

TABLE I

Disappearance of I^{131}-albumin and NaI^{131} from a normal leg *

Subject	Age	Sex	BSA	I^{131}-alb. disapp. $T_{\frac{1}{2}}$	I^{131}-alb. appearance after			I^{131}-alb. diffusion after			Urinary excretion†	Thyroid uptake‡	Serum ppt. act.	NaI^{131} disapp. $T_{\frac{1}{2}}$
					6	24	48	72 hrs	0	6	24	48 hrs				
	yrs		m^2	*hrs*	*% dose/L. plasma*				cm^2			cm^2			*%*	*min*
R. H.	42	M	1.75	42	1.6	4.9	7.7	7.5	3.8	4.5	6.2	8	3.5	1.7	>97	9
N. B.	42	M	1.72	35	2.1	5.4	7.2	7.4	2.5	5.3	14	17	4.3	2.6	>98	18
B. M.	36	F	1.61	39									3.2	1.7		11§
A. R.	28	M	1.65	24	2.3	8.0	8.7	9.1	2.5	4.5	10	13	3.6	2.8	>97	
A. M.	55	F	1.79	48	2.0	4.0	5.8	6.2					5.7	1.5	>97	12
A. L.	61	M	1.82	42	1.8	5.8	7.2	7.4	3.1	6.2	13	14	4.6	1.4	>96	
E. S.	58	F	1.60	30	2.9	7.6	8.6	8.6	5.3		7.1	8.0	3.8	1.9		7§
R. B.	45	M	1.74	26	2.4	6.8	7.2	7.1					5.2	2.1	>97	10
L. P.	43	M	1.70	36	2.5	5.8	7.0	6.9	5.3	6.2	10	13	3.5	1.0	>97	
C. F.	46	F	1.58	20	2.7	8.2	9.0	8.6	3.1		9.1	11	4.8	2.2	>96	13
M. S.	53	F	1.75	32					3.1	5.3	11		4.1	1.8		15§
L. W.	52	M	1.67	38	2.8	6.5	8.4	7.9					3.9	1.4	>98	
R. S.	28	F	1.69	18	2.5	7.2	7.7	7.4	4.5	7.1	13	15	5.2	2.1	>97	
B. K.	52	M	1.74	45	2.1	6.0	7.5	7.4					4.6	1.6	<97	10
F. S.	41	F	1.54	24	3.2	9.4	10.5	10.2	4.5	8	20	23	4.2	3.1	>96	14
Mean	45.5		1.69	33.4	2.4	6.6	7.9	7.8	3.8	5.9	11.3	13.6	4.3	1.9	> 96.9	11.9
SD	±9.9		±0.08	±9.3	±0.5	±1.5	±1.2	±1.1	±1.1	±1.2	±4.0	±1.5	±0.7	±0.6	> ±0.6	±3.2

* I^{131}-alb. disapp. =disappearance of I^{131}-albumin from the limb; I^{131}-alb. diffusion =area of diffusion of I^{131}-albumin about the injection site; Serum ppt. act. =% of total plasma radioactivity precipitated by 10% trichloroacetic acid.
† Per cent of the I^{131}-albumin dose excreted in the urine per day.
‡ Per cent of the I^{131}-albumin dose taken up by the thyroid per day.
§ $T_{\frac{1}{2}}$ of $Na^{24}Cl$.

TABLE II

Disappearance of I^{131}-albumin and NaI^{131} from a lymphedematous and opposite normal limb

Subject	Age	Sex	BSA	Cause of lymph-edema	Limb	I^{131}-alb. disapp. $T_{\frac{1}{2}}$	I^{131}-alb. appearance after				I^{131}-alb. diffusion after				Urinary excre-tion*	Thyroid uptake†	Serum ppt. act.	NaI^{131} disapp $T_{\frac{1}{2}}$
							6	24	48	72 hrs	0	6	24	48 hrs				
	yrs		m^2			*hrs*	*% dose/L plasma*				cm^2		cm^2				*%*	*min*
E. A.	57	F	1.85	Radical mastectomy	Control	27	1.0	2.9	3.8	4.1	3.1	5.3	13	15	2.1	1.1	>96	7‡
					Edema§	140					2.5	4.5	10	15				8
M. G.	65	F	1.78	Radical mastectomy	Control	26	1.4	3.4	4.0	4.3					3.0	1.2	>97	6
					Edema	92												7
A. A.	54	F	1.82	Radical mastectomy	Control	21	1.2	3.2	3.6	3.9	3.8		7.1	8.0	2.4	0.8	>96	10‡
					Edema	90					3.8		9.1	11				10
D. B.	59	F	1.74	Radical mastectomy	Control	22									2.8	0.7		15
					Edema	101												14
J. D.	62	F	1.83	Radical mastectomy	Control	27	1.3	3.2	4.0	4.3	4.5	5.3	9.1	11	2.2	0.9	>97	11
					Edema	118					3.8	4.5	8.0	9.1				9
E. P.	32	F	1.65	Radical groin resection	Control	33	1.7	3.4	4.4	4.9	3.1	6.2	10	13	2.7	1.0	>96	12
					Edema	65					2.5	6.2	14	15				13
Mean	54.8		1.78			26.0					3.6	5.6	9.8	11.8				10.2
SD	±11.8		±0.08		Control	4.3	1.3	3.2	3.9	4.3	±0.7	±0.5	±2.4	±3.0	2.5	1.0	>96.4	±3.3
Mean							±0.3	±0.2	±0.3	±0.4					±0.4	±0.2	> ±0.5	
S.D.					Edema	101.0 ±25.7					3.2 ±0.8	5.1 ±1.0	10.3 ±2.6	13.8 ±3.2				10.2 ±2.8

* Per cent of I^{131}-albumin dose excreted in the urine per day.
† Per cent of I^{131}-albumin dose taken up by the thyroid per day.
‡ $T_{\frac{1}{2}}$ of $Na^{24}Cl$.
§ Lymphedematous.

ance) and for decay of the isotopes. In repeat studies in cardiac patients and in all studies on paired extremities, the administered dose and volume of the radioactive ion was the same. The sites of injection were also controlled.

Two types of collimation were employed to limit the detected radiation to the monitored areas. For the lower extremities, a shield of lead bricks, 2 inches thick, was constructed to provide a square opening (approximately 100 cm^2) over which the injection site could conveniently be placed in front of the radiation detector, which was recessed 15 cm below the opening of the shield. For monitoring the thyroid gland and the injection site in the upper extremities, a conical lead shield, 2 inches thick, with a round opening (approximately 175 cm^2) into which the radiation detector was recessed 40 cm, was used.

Duplicate determinations of the monitored radioactivity checked within 2 per cent. Tissue background radiation arising from circulating I^{131}-albumin at the injection site was assumed to be equal to that of a similar area in the opposite extremity.

RESULTS

The results are summarized in Tables I through VII.

Removal of I^{131}-albumin from the normal limb. The disappearance rate of injected labeled albumin from the subcutaneous tissue of a normal leg is illustrated in Figure 1. For the first 4 to 8 hours following the injection of I^{131}-albumin there was a relatively rapid decrease in radioactivity over the injected area in 10 of the 15 cases. During this period 10 to 28 per cent of the injected dose disappeared. Thereafter the radioactivity decreased more slowly. The half-time of disappearance ($T_{\frac{1}{2}}$), calculated from the slower exponential component of the disappearance curve, averaged 33.4 hours and ranged from 18 to 48 hours. In contrast to these values, the $T_{\frac{1}{2}}$ of NaI^{131} from the subcutaneous tissue of the same subjects averaged 11.9 minutes and ranged from 7 to 18 minutes. There was no consistent relationship between the $T_{\frac{1}{2}}$ of I^{131}-labeled albumin and that of NaI^{131}.

Repeated scanning over the site of injection revealed that I^{131}-albumin remained within the monitored area, although some diffusion occurred. Immediately after injection, the area occupied by the injected activity averaged about 3.8 cm^2, whereas 24 and 48 hours after the injection it averaged 11.3 and 13.6 cm^2, respectively. The relationship between the $T_{\frac{1}{2}}$ and the area of diffusion of the

TABLE III

Disappearance of I^{131}-albumin and NaI^{131} from paired normal limbs

Subject	Age	Sex	BSA	Limb*	I^{131}-alb. disapp. $T_{\frac{1}{2}}$	NaI^{131} disapp. $T_{\frac{1}{2}}$
	yrs		m^2		*hrs*	*min*
L. M.	52	F	1.77	R. F.	19	6
				L. F.	20	5
A. C.	57	F	1.84	R. F.	25	18†
				L. F.	27	20
C. F.	49	F	1.81	R. F.	25	10
				L. F.	25	11
M. C.	54	F	1.75	R. F.	22	15
				L. F.	22	13
E. B.	34	F	1.68	R. L.	31	
				L. L.	29	
E. K.	42	F	1.72	R. L.	34	14
				L. L.	32	14
N. B.	37	F	1.64	R. L.	28	9†
				L. L.	38	8
Mean	46.4		1.74	R. Ext.	26.3	12.0
SD	±8.9		0.08		±5.2	±4.4
Mean				L. Ext.	27.6	11.8
SD					±6.1	±5.2

* R.F. = right forearm; L.F. = left forearm; R.L. = right leg; L.L. = left leg; R. Ext. = right extremity; L. Ext. = left extremity.

† $T_{\frac{1}{2}}$ of $Na^{24}Cl$.

TABLE IV

Disappearance of I^{131}-albumin in edema due to venous obstruction

Subject	Age	Sex	BSA	Cause of edema	Leg	I^{131}-alb. disapp. $T_{\frac{1}{2}}$	I^{131}-alb, appearance after				I^{131}-alb, diffusion after				Urinary excretion*	Thyroid uptake†	Serum ppt. act.
							6	24	48	72 hrs	0	6	24	48 hrs			
	yrs		*m²*			*hrs*	*% dose/L plasma*				*cm²*		*cm²*				*%*
S. T.	59	F	1.62	Femoral vein thrombosis	Control	32	2.5	7.6	8.2	9.0					3.2	2.1	>97
					Edema	20											
E. C.	42	F	1.58	Femoral vein thrombosis	Control	41	2.3	7.5	8.7	9.2	4.5	7.1	13	15	4.5	1.8	>96
					Edema	18					3.1	8	17	23			
H. S.	48	M	1.92	Femoral vein thrombosis	Control	27	1.9	5.2	5.7	5.9					4.8	1.9	>97
					Edema	12											
A. S.	55	F	1.88	Popliteal vein thrombosis	Control	34					2.5	6.2	13	17	4.1	1.5	
					Edema	12					2.5	8	20	30			
D. M.	64	M	1.75	Inf. vena caval block	Edema	16	3.1	6.0	6.6	6.2					1.8	3.5	>98
R. M.	61	M	1.78	Inf. vena caval block	Edema	18	2.6	5.6	6.0	5.9	3.1	4.5	13		2.0	2.7	>96
A. R.	42	M	1.72	Sup. vena caval block	Control‡	36	2.0	6.4	7.0	7.7					3.3	2.9	>96
					Edema§	17											
Mean	53.0		1.75		Control	34.0	2.4	6.4	7.0	7.3					3.4	2.3	>96.7
SD	±9.1		±0.1			±5.1											
Mean					Edema	16.1	±0.4	±1.0	±1.2	±1.5					±1.2	±0.7	> ±0.8
SD						±3.1											

* Per cent of I^{131}-albumin dose excreted in the urine per day.
† Per cent of I^{131}-albumin dose taken up by the thyroid per day.
‡ Nonedema, as legs.
§ Edematous forearm.

TABLE V

Disappearance of I^{131}-albumin in cardiac edema

Subject	Age	Sex	BSA	Diagnosis*	Edema grade	Venous pressure	I^{131}-alb. disapp. $T_{\frac{1}{2}}$	I^{131}-alb. appearance after				I^{131}-alb. diffusion after			Urinary excretion†	Thyroid uptake‡	Serum ppt. act.
								6	24	48	72 hrs	0	6	24 hrs			
	yrs		m^2			*mm H_2O*	*hrs*	*% dose/L. plasma*				*cm^2*					%
F. R.	62	M	1.75	ASHD	4+	205	18	2.5	6.6	7.0	6.8	3.1	8	25	0.8	3.4	>97
M. M.	47	M	1.71	ASHD	4+	305	12	4.4	7.8	8.0	8.0	2.5	7.1	20	1.7	2.5	>96
M. L.	60	F	1.72	ASHD	3+	235	24	2.0	5.3	5.0	5.8	3.4		17	2.1	3.0	>97
E. J.	44	M	1.67	Hyp., ASHD	3+	205	10	5.2	9.6	9.4	9.0				1.9	2.3	>98
J. B.	64	M	1.70	Hyp., ASHD	4+	315	30	2.1	5.4	6.9	6.6				0.9	3.5	>96
W. B.	52	M	1.68	HCVD	3+	325	13	3.4	5.6	6.0	6.0				1.2	2.8	>96
A. S.	59	M	1.62	HCVD	4+	255	28					3.8	8	20			
M. A.	39	M	1.69	HCVD	4+	180	15	3.2	5.9	6.2	5.9				1.6	2.2	>97
M. F.	56	M	1.84	HCVD	3+	285	14					4.5	13	37	1.0	3.3	
G. L.	45	M	1.64	RHD MI, MS	4+	235	12	3.5	5.4	5.1	5.0	4.5	9.1	26	1.1	2.9	>96
C. J.	41	F	1.51	RHD, MI, MS	3+	275	12	4.4	9.5	9.0	8.8				2.2	3.1	>98
W. G.	43	M	1.58	RHD, AI, MI	4+	205	11	4.8	7.5	7.3	7.2	2.5	4.5	11	1.8	2.5	>97
W. M.	45	F	1.73	RHD., MI	4+	295	16	3.0	5.6	5.8	5.7	3.1	4.5	13	2.0	2.7	>97
R. L.	23	M	1.82	Pericard.	3+	340	15	3.1	5.7	6.1	6.0	3.1	10.1	30	0.6	2.8	>96
F. B.	41	M	1.60	Pul. sten.	3+	240	20	2.2	6.5	7.2	7.0	3.1	10	28	1.2	3.0	>97
Mean	48.1		1.68			260	16.7	3.4	6.6	6.9	6.7	3.4	8.3	22.9	1.4	2.8	>96.8
SD	±10.9		0.09			±49.9	±6.2	±1.1	±1.5	±1.3	±1.2	±0.7	±2.7	±8.4	±0.5	±0.4	>±0.8

* ASHD =arteriosclerotic heart disease; Hyp. =hypertensive; HCVD =hypertensive cardiovascular disease; RHD =rheumatic heart disease; Pericard. =pericarditis, chronic constrictive; MI = mitral insufficiency; AI =aortic insufficiency; MS =mitral stenosis; Pul. sten. =pulmonic stenosis.

† Per cent of I^{131}-albumin dose excreted in the urine per day.

‡ Per cent of I^{131}-albumin dose taken up by the thyroid per day.

TABLE VI

Disappearance of I^{131}-albumin and $Na^{24}Cl$ in cardiac edema following therapy

Subject	Treatment	Edema grade	Venous pressure	Decholin circ. time	I^{131}-alb. disapp. $T_{\frac{1}{2}}$	Na^{24} Cl disapp.
			mm H_2O	*sec*	*hrs*	
M. A.	None	4+	180	35	15	54
	After 8 days	0	80	14	31	15
M. F.	None	3+	285	38	12	62*
	After 7 days	0	75	15	25	14
G. L.	None	4+	235	33	12	
	After 7 days	0	100	24	40	
C. J.	None	3+	275	45	12	67
	After 8 days	0	185	40	24	60
W. G.	None	4+	205	31	11	27
	After 8 days	0	95	23	44	19
R. L.	None	3+	340	46	15	56
	After 15 days	0	90	13	24	16
F. B.	None	3+	240	34	20	52*
	After 7 days	0	145	27	34	41
Mean	None		251	37.4	13.9	53.0
SD			±54	±5.9	±3.1	±13.9
Mean	After		110	22.1	31.7	27.5
SD			±40	±9.5	±8.1	±18.9

* $T_{\frac{1}{2}}$ of NaI^{131}.

injected activity was inconsistent. Scans of the limbs also showed that the radioactivity throughout the injected limb, except for the injection site, was comparable to that in the opposite control limb and was not accompanied by a detectable increase of radioactivity over the regional lymph nodes.

As I^{131}-albumin disappeared from the site of injection, the radioactivity in the blood increased progressively in the first 18 to 36 hours and

TABLE VII

Disappearance of I^{131}-albumin in hypoproteinemic edema

Subject	Age	Sex	BSA	Diagnosis	Edema grade	Serum total protein	Serum albumin	I^{131}-alb. disapp. $T_{\frac{1}{2}}$
	yrs		*m^2*			*g %*	*g %*	*hrs*
C. S.	48	M	1.60	Cirrhosis	3+	6.9	2.4	10
C. C.	55	M	1.72	Cirrhosis	3+	4.2	1.9	13
M. C.	51	F	1.52	Cirrhosis	2+	5.4	1.8	20
R. C.	62	M	1.79	Cirrhosis	3+	5.0	1.8	16
M. R.	49	M	1.68	Cirrhosis	3+	5.2	2.2	14
A. P.	45	F	1.57	Cirrhosis	4+	4.8	1.8	17
C. M.	54	F	1.58	Nephrotic syndrome	4+	5.5	0.9	17
E. N.	38	M	1.74	Nephrotic syndrome	4+	5.1	1.3	18
T. R.	23	M	1.65	Nephrotic syndrome	4+	5.2	1.9	15
C. A.	65	F	1.82	Nephrotic syndrome	4+	5.7	1.2	12
Mean	49.0		1.67			5.3	1.7	15.2
SD	±12.0		±0.1			±0.7	±0.5	±3.0

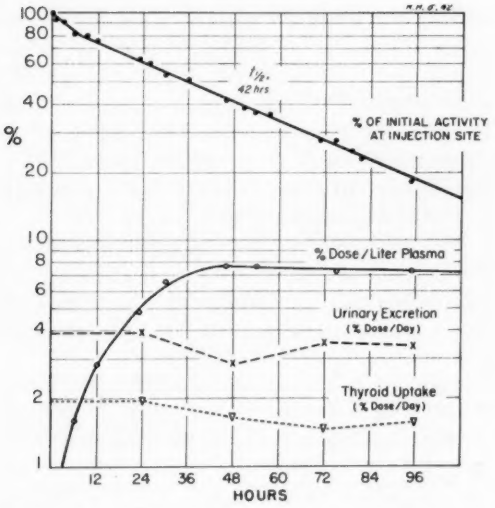

FIG. 1. THE DISAPPEARANCE OF I^{131}-ALBUMIN FROM THE SUBCUTANEOUS TISSUE OF A NORMAL LEG.

reached a plateau in 48 to 72 hours. The buildup of radioactivity in the plasma was more rapid in those subjects with a short than in those with a long $T_{\frac{1}{2}}$ of I^{131}-labeled albumin. Of the total activity in each plasma sample, more than 96 per cent was contained on the protein fraction precipitated by 10 per cent trichloroacetic acid.

Thyroid and urine radioactivities averaged, respectively, 1.9 and 4.3 per cent of the injected dose per day.

Removal of I^{131}-albumin from the lymphedematous limb. The clearance of I^{131}-albumin and NaI^{131} from the subcutaneous tissue of a lymphedematous limb due to a radical mastectomy and that of the opposite normal limb are compared in Figures 2 and 3.

In six patients I^{131}-albumin disappeared at a significantly slower rate from the lymphedematous limb than from the control limb ($p < 0.01$). The $T_{\frac{1}{2}}$ from the involved limb averaged 101 hours and ranged from 65 to 140 hours as compared to an average of 26 hours and a range of 21 to 33 hours from the opposite normal limb. Some diffusion of the injected albumin occurred in the tissue but was not appreciably different in the two limbs. In contrast to these results, the disappearance of NaI^{131} and $Na^{24}Cl$ did not differ significantly in the lymphedematous and opposite normal limb ($p > 0.9$) and was approximately 150 to 600 times more rapid than the disappearance of I^{131}-albumin. In control studies of paired normal limbs the disappearance of labeled albumin as well as of NaI^{131} and $Na^{24}Cl$ was approximately the same ($p = 0.4$ and $p = 0.8$, respectively).

Following the subcutaneous injection of I^{131}-albumin, radioactivity, more than 96 per cent of which was precipitable with 10 per cent trichloroacetic acid, appeared in the plasma. The average urinary excretion of radioactivity was 2.5 per cent per day, whereas the radioactivity taken up by the thyroid gland averaged 1 per cent per day.

Removal of I^{131}-albumin in edematous limbs caused by venous occlusion. The disappearance of I^{131}-albumin from an edematous limb caused by phlebothrombosis is compared with that of the opposite normal limb in Figure 4.

The disappearance of I^{131}-albumin in edema caused by venous obstruction in contrast to that caused by lymphatic obstruction was significantly increased as compared to the disappearance of I^{131}-albumin in control limbs ($p < 0.01$). In four subjects with venous obstruction, the mean disappearance time of I^{131}-albumin in the edematous limb was 16 hours, whereas in the opposite normal limb it was 34 hours. The diffusion of radioactivity from the subcutaneous injection site also appeared to be greater in the edematous than in the control limb. It is noteworthy that the re-

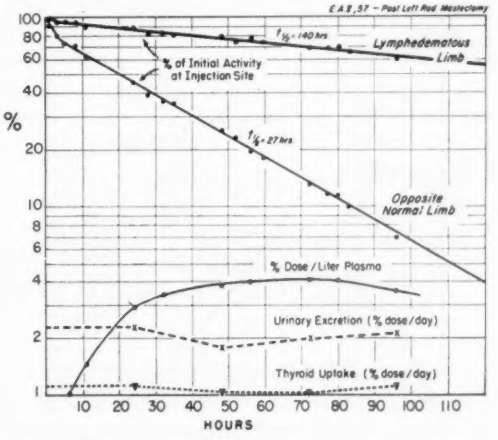

FIG. 2. THE DISAPPEARANCE OF I^{131}-ALBUMIN FROM THE SUBCUTANEOUS TISSUE OF A LYMPHEDEMATOUS FOREARM DUE TO A RADICAL MASTECTOMY FOR CARCINOMA OF THE BREAST AS COMPARED TO THAT OF THE OPPOSITE NORMAL FOREARM.

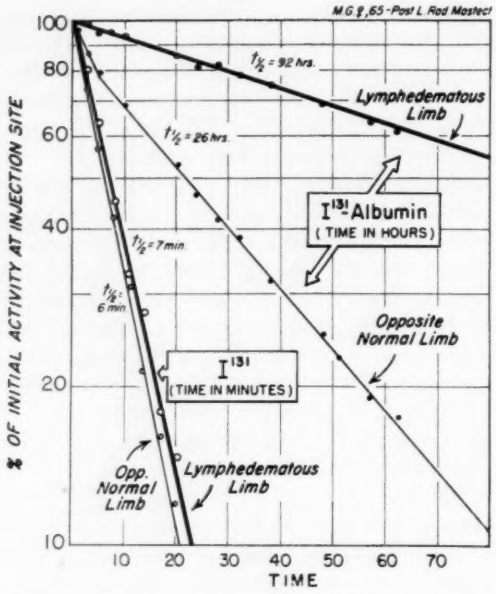

FIG. 3. THE DISAPPEARANCE OF I^{131}-ALBUMIN AND NaI^{131} FROM THE SUBCUTANEOUS TISSUE OF A LYMPHEDEMATOUS FOREARM DUE TO A RADICAL MASTECTOMY AND FROM THE OPPOSITE NORMAL FOREARM.

moval of I^{131}-albumin from the upper extremities in Patient A.R., with superior vena caval block, was unimpaired even though the antecubital venous pressure was 465 mm H_2O. The appearance

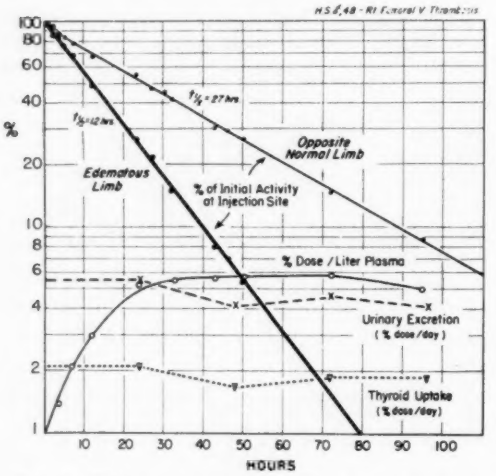

FIG. 4. THE DISAPPEARANCE OF I^{131}-ALBUMIN FROM THE SUBCUTANEOUS TISSUE OF AN EDEMATOUS LEG CAUSED BY FEMORAL VEIN THROMBOSIS AS COMPARED TO THAT OF THE OPPOSITE NORMAL LEG.

of radioactivity in the plasma, urine and thyroid gland was similar to that observed in the control group.

Removal of I^{131}-albumin in cardiac edema. The clearance of I^{131}-albumin and $Na^{24}Cl$ from the subcutaneous tissue of the leg during and following recovery from heart failure in a hypertensive patient is illustrated in Figure 5.

The disappearance and appearance curves of I^{131}-albumin in patients with cardiac edema were similar to those in normal subjects. However, the $T_{\frac{1}{2}}$, which averaged 16.7 hours and varied

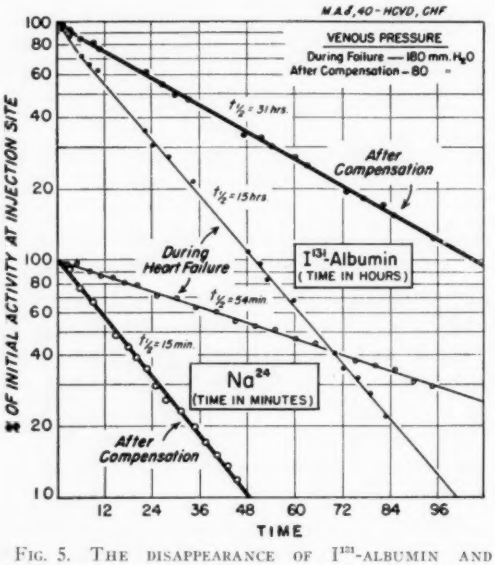

FIG. 5. THE DISAPPEARANCE OF I^{131}-ALBUMIN AND $NA^{24}CL$ FROM THE SUBCUTANEOUS TISSUE OF A LEG OF A CARDIAC PATIENT DURING AND FOLLOWING RECOVERY FROM CONGESTIVE HEART FAILURE.

from 11 to 30 hours, was significantly shorter in the cardiac edematous than in the normal leg ($p < 0.01$). The buildup of activity in the blood also appeared to be more rapid in patients with cardiac edema than in normal subjects, although there was an inconsistent relationship between the actual level of radioactivity measurable in the plasma and the amount of I^{131}-albumin which had disappeared from the site of injection. The radioactivity excreted in the urine was significantly less ($p < 0.01$), whereas the radioactivity appearing in the thyroid gland was significantly greater ($p < 0.01$) than the values obtained in the control group. The area of diffusion of the injected

activity in the subcutaneous tissue was significantly greater in the cardiac edematous than in the normal limb ($p < 0.01$). Twenty-four hours following injection of the I^{131}-albumin, radioactivity of the injection site covered an average area of 22.9 cm^2 in edematous cardiac patients as compared to 11.3 cm^2 in normal subjects. Although the average rate of removal of $Na^{24}Cl$ in cardiac edema was about 50 times more rapid than that of I^{131}-albumin, it was significantly slowed, being about one-fourth as rapid as that in normal subjects ($p < 0.01$).

In seven subjects who were restudied after cardiac compensation, the removal of I^{131}-albumin from the subcutaneous tissue of the legs decreased noticeably toward the normal range. The $T_{\frac{1}{2}}$ averaged 31.7 hours as compared to 13.9 hours before treatment. The clearance of Na^{24} also returned toward normal after treatment, but in contrast to that of I^{131}-albumin, it was considerably more rapid after compensation than it had been during congestive failure. The $T_{\frac{1}{2}}$ of Na^{24} before treatment averaged 53 minutes, whereas after treatment it averaged 28 minutes. The return of Na^{24} clearance to the normal range after treatment was associated with "complete" compensation as indicated by the disappearance of peripheral edema and a decrease in venous pressure and circulation time to normal levels. However, normal clearance values for I^{131}-albumin occurred without "complete" compensation, but it was consistently associated with the disappearance of edema.

Removal of I^{131}-albumin in hypoproteinemic edema. The disappearance of I^{131}-albumin from the subcutaneous tissue of the legs of patients with hypoproteinemic edema caused by cirrhosis or nephrotic syndrome was significantly more rapid than that in the control group ($p < 0.01$). The $T_{\frac{1}{2}}$ averaged 15.2 hours and ranged from 10 to 20 hours.

DISCUSSION

I^{131}-albumin appeared to have a high degree of stability following injection into the subcutaneous tissue. The existence of a firm bond between the radioiodine and albumin molecules in the tissue is supported by the observation that the disappearance of I^{131}-albumin from the tissues was considerably slower than that of inorganic I^{131}. The amount of radioiodine excreted in the urine and taken up by the thyroid gland following the subcutaneous injection of the labeled albumin was small and consistent with the metabolic breakdown of plasma proteins, and over 96 per cent of the plasma radioactivity was contained on the protein fraction precipitated by trichloroacetic acid and was accounted for in the albumin fraction separated by electrophoresis.

The removal of labeled albumin from the resting limb was slow but occurred at an exponential rate. The explanation for the increased clearance of the labeled protein in the first 4 to 8 hours after injection is not clear but might be due to the injection of some radioactivity directly into the subcutaneous lymphatic capillaries or to a temporary injury to the lymphatic capillaries by the injection. Some dissociation of I^{131} from the albumin molecule has been reported to occur in certain batches of radioiodinated serum albumin (16). However, it is unlikely that the clearance of unbound I^{131} would account for the relatively rapid fall-off of activity at the injection site during the first 4 or more hours, since the removal of inorganic I^{131} was virtually complete in 1 hour.

The appearance of radioactivity in the blood did not parallel the disappearance of radioactivity from the site of injection even when appropriate corrections were made for the radioactivity which had been excreted in the urine or taken up by the thyroid gland. The dissimilarities in the disappearance and appearance curves are not surprising, since the level of radioactivity in the blood at a given time presumably is influenced not only by the amount of radioactivity appearing in the blood from the injection site but also by the volume of distribution of the radioactivity, which increases as the labeled albumin equilibrates with extravascular protein.

The marked reduction in the clearance of I^{131}-albumin from the subcutaneous tissue in lymphedema suggests that the principal route of removal of extravascular protein in man is through the lymphatic vessels and that the direct passage of interstitial protein across blood capillaries into the blood stream is negligible in such a limb. Studies with I^{131}-albumin in animals also support this view (17). The finding also suggests that the rate of disappearance of labeled albumin might be employed as an index of lymphatic "function," including flow, since it is likely that protein is ab-

sorbed by the lymphatic vessels along with a relatively fixed amount of interstitial fluid. In animal studies, Drinker and Field have concluded that the absorption of protein by the lymphatics is accompanied by salt and water in the same concentrations that exist in the tissue fluid (1).

The present observations that the disappearance of Na^{24} or I^{131} from subcutaneous tissue is much more rapid that that of I^{131}-albumin, that it is comparable in lymphedematous and normal limbs, and that it is influenced by the degree of cardiac compensation, are in line with the evidence that inorganic ions are principally cleared from the interstitial fluid by direct diffusion through the blood capillaries into the blood stream (18–21). The findings are also consistent with reports that the rate of disappearance of inorganic ions from the tissues may be a useful index of blood flow, especially through the capillaries (18–20).

With the exception of lymphatic edema, the disappearance of I^{131}-albumin in edema caused by other conditions such as venous obstruction, congestive heart failure, and hypoproteinemia was significantly more rapid than the removal of I^{131}-albumin from normal limbs. It is unlikely that the differences in albumin removal were due to differences of diffusion of the injected protein in the subcutaneous tissue, since the I^{131}-albumin disappeared at an exponential rate. It is also improbable that differences shown by the different groups in thyroidal and renal clearances of radioiodide liberated from degraded I^{131}-albumin influenced the disappearance of the injected albumin.

An increase in permeability to protein in the blood capillaries might also promote the removal of I^{131}-albumin by the blood stream. However, evidence for such a change is lacking in cardiac and nephritic edema, since the interstitial protein concentration in these conditions is only a small fraction of the plasma protein concentration and no greater than the protein content of interstitial fluid in normal limbs (22, 23). Furthermore, Ross and Walker have recently presented findings which indicate a decrease rather than an increase in capillary permeability to protein in congestive heart failure (24). Other present observations which argue against the blood stream as the major route of removal of subcutaneously injected albumin in cardiac edema are those showing that the clearance of I^{131}-albumin changed in an opposite direction to that of NaI^{131} or $Na^{24}Cl$ following recovery from heart failure.

These results are consistent with the presence of an increased lymphatic flow in cardiac edema. They also appear to indicate that edema fluid in heart failure is not "stagnant," but actually exchanges with the fluid and salt in the blood and circulates through the lymphatics at a fairly constant rate and more rapidly than does normal interstitial fluid.

As opposed to the present findings, McMaster has concluded from the local spread of vital dyes injected intradermally that lymphatic flow, although increased in nephritic edema, was negligible or absent in cardiac edema (10). Threefoot, using similar methods of study, has indicated a reduction in lymphatic flow within the skin of all edematous extremities (11). Since leakage of the vital dye from the cutaneous lymphatics was reported to occur in both these studies, it might have influenced the results of the experiments. Furthermore, the interpretation of these studies was made from the distribution and not from the actual rate of removal of the dye from the tissues.

In addition to the methods of measurement, other factors which might account for differences in results between those and the present studies include the age of the patients studied, duration of the edema, and differences in the cutaneous as compared with the subcutaneous lymphatic flow. In the current study the patients were younger and had a shorter history of heart failure than the patients studied by McMaster.

In edema, especially of long duration, a proliferation of interstitial tissue may occur and interfere with lymphatic drainage. This factor and differences in interstitial pressure may account for the variations in the clearance of labeled albumin from edematous tissues. It likewise appears from the observations in edematous states, especially in heart failure and superior vena caval block syndrome, that the interstitial pressure is an extremely important determinant of lymphatic flow, even when marked hypertension exists in the veins into which the main lymphatics drain.

SUMMARY

The disappearance of human serum albumin labeled with radioiodine from the subcutaneous

tissue of the forearm or leg described an exponential slope and was followed by the appearance of radioactive albumin in the blood. The rate of removal of I^{131}-albumin from the tissues was significantly reduced in edema caused by lymphatic obstruction, but significantly increased in edema caused by venous obstruction, congestive heart failure or hypoproteinemia. The disappearance of $Na^{24}Cl$ or of NaI^{131} from the tissues was considerably more rapid than that of I^{131}-albumin and was not significantly altered in lymphatic edema, although it was significantly reduced in cardiac edema. Following treatment of the heart failure, the clearance of labeled protein and inorganic ions changed in opposite directions and returned to or toward normal values.

These findings indicate that I^{131}-albumin injected into the subcutaneous tissue is removed mainly by lymphatic vessels and suggest that lymphatic flow is reduced in lymphedema but is increased in cardiac and other types of edema.

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ION ASSOCIATION. IV. EFFECT OF SODIUM SULFATE INFUSION ON RENAL CLEARANCE AND BODY RETENTION OF INJECTED RADIOSTRONTIUM IN DOGS *

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The removal of toxic metals from the body by administering appropriate complexing agents has been the subject of intensive study in recent years. The agents employed have usually been organic chelating compounds, chosen because of their affinity for the toxic metal to be removed. This approach has been highly successful in the case of some metals but of limited success in the case of radioactive strontium. The chemical similarity of strontium to calcium renders difficult the search for a compound which will remove strontium in preference to calcium (3–5).

Since calcium and strontium are apparently handled in similar fashion by the kidney (6–11), increased calcium excretion is associated with increased strontium excretion. Therefore, a more successful approach has been the administration of agents known to increase calcium excretion, including calcium salts, ammonium chloride, sodium citrate, parathyroid extract, and a variety of anions which chelate both calcium and strontium (8, 12–30). Even when these substances are administered, a major portion of the filtered loads of both ions continues to be reabsorbed by the renal tubules. Consequently, it is difficult to achieve very high rates of calcium or strontium excretion by these means. Although retention of radiostrontium may be considerably reduced by administering some of these agents either prior to or simultaneously with the isotope, therapy instituted after exposure removes only a small amount.

The use of external (31) or peritoneal (32) dialysis in dogs has been somewhat more successful, 30 to 40 per cent of the dose being removed by therapy instituted after 1 hour of exposure (31). However, the amount of isotope mobilized was only slightly greater than the amount estimated to be present in the extracellular fluid. Therapy instituted after 12 hours removed less than 12 per cent of the remaining radiostrontium (31).

The marked effect of sulfate infusion on the renal excretion of both calcium and magnesium (33, 34) suggested the use of sodium sulfate in order to accelerate strontium excretion. The mechanism by which sulfate augments the excretion of alkaline earth cations, discussed in a previous paper (34), is apparently related to the formation of electrostatic ion-pairs, which differ from metal-chelate compounds in that covalent bonding is not thought to occur (35). Although the dissociation constant of the strontium sulfate ion-pair has apparently not been reported, it is probably close to the dissociation constants of the calcium sulfate and magnesium sulfate ion-pairs, both of which are approximately 0.06 M at ionic strength 0.16 M (36).

Therefore strontium ions, in common with calcium and magnesium ions, may be poorly reabsorbed by the renal tubules when paired electrostatically with sulfate ions. In this manner, renal excretion of strontium may be accelerated by sulfate infusion.

Even the complete removal of radiostrontium from the extracellular fluid by means of renal excretion would be of little benefit unless mobilization of isotope retained in the skeleton and in the tissues also occurred. It has been shown repeatedly that the fraction of the remaining isotope in the blood stream falls rapidly without therapy (6, 8, 10–12, 21, 37, 38). Schulert and coworkers (39) have recently reported that the soft tissues contain approximately as much isotope as the skeleton during the first few weeks.

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TABLE I

Summary of experimental procedures

Dog	Wt	Isotope	Dose	Period of infusion	Rates of infusion	Composition of infusion						Plasma SO_4 range
						Ca	Mg	K	SO_4	Acetate	Mannitol	
	kg		*μc*	*hrs after injection*	*ml/min*	*mmoles/L*						*mmoles/L*
D	9	Sr^{89}	200	14–17	13	0, 5*	2	16	100	0	0	20–23
E	11	Sr^{89}	200	168–188	13	3	3	16	100	0	0	13–24
M	23	Sr^{89}	200	72–80	25	3	3	0, 16*	93	0	0	13–27
Q	11	Sr^{85}	50	14–24	20	2	2	16	100	0	0	17–26
R1	19	Sr^{85}	50	18–25	16	4	4	0	90	18	0	8–18
R2	19	Sr^{85}	50	41–52	7	0	0	0	100	20	0	6–10
S	11	Sr^{85}	50	4–8	20	0	5	0	100	0	0	17–20
T	11	Sr^{85}	37.5	20–24	10	0, 5*	2	16	100	0	0	
U	11	Sr^{85}	37.5	21–24	15–35	0, 5*	2	16	100	0	0	17–29
V	19	Sr^{85}	12	2.5–5	15–23	3	3	8	97	0	50	21–29

* In these experiments infusions of two different compositions were employed in the order indicated.

There are two ways in which an agent such as sulfate might mobilize skeletal deposits of radiostrontium. First, the removal of circulating isotope would lead to exchange between circulating calcium ions and radiostrontium atoms in the crystal lattice of bone. Secondly, the production of negative calcium balance and the reduced plasma concentration of free calcium ions would lead to some demineralization of bone. Similar considerations might apply to extraskeletal repositories of calcium and radiostrontium.

The present experiments were conducted to determine the effect of sulfate infusion on the renal clearance and bodily retention of radiostrontium in dogs. Because of varying nutrition (and possibly age), the individual dogs differed in the amount of radiostrontium retained before sulfate infusion and therefore in the percentage mobilized during the infusion. Nevertheless, a uniform relationship between radiostrontium clearance and sulfate excretion was observed, irrespective of the interval before therapy, the percentage of isotope remaining, or the amounts of calcium and magnesium in the infused solution. At high rates of infusion, renal tubular reabsorption of strontium almost ceased.

METHODS

Carrier-free isotopes of strontium,[1] Sr^{85} or Sr^{89}, obtained as $SrCl_2$ in HCl were appropriately diluted and injected intravenously into 9 female mongrel dogs weighing 10 to 18 kg. These animals, obtained from the pound, appeared to be healthy and fully grown but were in varying states of nutrition. They were placed in metabolism cages and urine and stool collections begun. The dogs were fed their usual diet of pellets. During the clearance measurements, urine was collected by indwelling urethral catheters, and each sample was analyzed separately. The technique of the clearance experiments and methods of analysis of calcium, creatinine and sulfate are described elsewhere (34, 40, 41). Stool samples were homogenized with water in a blender.

Table I summarizes the isotope dosages, periods of infusion, and infusion composition in each experiment. One dog (R) was given a single injection but was treated on two successive occasions; these two experiments are designated R1 and R2. In five experiments (D, Q, T, S and V) one or two control collection periods preceding sulfate infusion were obtained. In all ten, isotope excretion was measured during sulfate infusion. The duration of the infusion varied from 2.5 hours in the animal treated 2.5 hours after injection, to 20 hours in the dog treated 1 week after injection; in the others, the duration of the infusion averaged 6 hours.

Sr^{85} was determined in a scintillation counter equipped with a well. The radioactivity of 2-ml samples of plasma, urine, or homogenized stool suspension, counted for 10,000 counts or more, was compared with that of an aliquot of the injected solution, after correction for background. Sr^{89} was determined by evaporating aliquots to dryness on planchets fitted with absorbent paper discs. One ml of urine or an aliquot of the injected solution was mixed with 0.5 ml of normal plasma in the planchet in order to make self-absorption uniform; when plasma or stool suspension was analyzed, a 0.5 ml aliquot was mixed with 1 ml of water. Radioactivity was determined with an end-window GM counter. Duplicate aliquots of plasma samples yielded results differing by 1.5 per cent or less.

RESULTS

1. The increase in radiostrontium excretion during sulfate infusion. In five of the ten experiments, control periods were obtained during which

[1] Sr^{85} was obtained from Nuclear Science and Engineering Corporation, Pittsburgh, Pa. Sr^{89} was obtained from Oak Ridge National Laboratories.

TABLE II

Increase in radiostrontium excretion during first 30 to 60 minutes of sulfate infusion

	Urine flow		Sr excretion		
	Control	Sulfate	Control	Sulfate	Change
	ml/min	*ml/min*	*μc/min*	*μc/min*	
D	0.7	11.6	0.0186	0.506	27×
Q	0.7	10.6	0.0025	0.015	6×
S	1.1	9.9	0.0032	0.0916	29×
T	0.3	8.5	0.0011	0.018	16×
V	1.5	20.6	0.0054	0.208	39×

the dogs received either no intravenous fluids or a solution of creatinine at 1 ml per minute. Urine was obtained by indwelling catheter. The rate of isotope excretion in the first 30 to 60 minutes of sulfate infusion was 6 to 40 times greater than that in the control periods (Table II). This increment cannot be attributed to washout of concentrated urine from the renal tubules and pelvis, since urinary strontium concentration either increased or fell only slightly. In one additional dog, infusion of hypertonic sulfate solution followed infusion of hypertonic glucose (Table III). Despite falling urine flow, excretion of strontium (as well as calcium and magnesium) rose many fold when sulfate was administered.

In all of the dogs, the increase in radiostrontium excretion following infusion of sulfate led to a progressive decline in plasma radioactivity. Consequently, isotope excretion rate fell in subsequent collection periods. The effectiveness of sulfate (or of any other agent) in removing radiostrontium from the extracellular fluid is better evaluated by the change in isotope clearance, which in theory should be independent of isotope concentration.

2. The clearance of radiostrontium during sulfate infusion. In nine of the ten experiments, the renal clearances of radiostrontium, creatinine and sulfate were determined simultaneously during sulfate infusion. Strontium clearance was calculated without correction for protein-binding (see below). In normal dogs or humans, strontium clearance calculated in this manner has been reported to be two to five times calcium clearance, or approximately 3 ml per 100 ml of glomerular filtrate (GF) (6, 7, 11, 23). In 14 collection periods in six normal dogs undergoing water, glucose or mannitol diuresis, radiostrontium clearance averaged 5 ml per 100 ml of GF. This value represents the tubular reabsorption of over 90 per cent of the filtered isotope.

During sulfate infusion, strontium clearance increased to unprecedented levels, reaching values over 60 ml per 100 ml of GF. The relationship between radiostrontium clearance and urinary sulfate excretion is depicted in Figure 1. Both parameters are expressed per 100 ml of GF in order to compare animals of different size. Despite considerable variation in the size of the dogs and in the interval between isotope administration and start of therapy (see Table I), a uniform relationship is observed, which appears to be linear. This relationship resembles that found between calcium clearance and sulfate excretion in dogs, reported previously (34). A similar linear relationship is seen when radiostrontium clearance is plotted against plasma sulfate concentration.

3. The effect of other ions on the strontiuretic action of sulfate. Since it is known that administration of calcium leads to increased strontium excretion (8, 18, 24), it was important to determine that the increase in radiostrontium clearance was not the result of the calcium present in most of

TABLE III

Effect of sulfate infusion on excretion of alkaline earth cations at decreasing urine flow in Dog H (13.7 kg)

Time	Plasma			Urine					C_{Ca}/GFR ratio	C_{Mg}/GFR ratio	C_{Sr}/GFR ratio
	Ca	Mg	Sr^{85}	Flow	GFR	Ca	Mg	Sr			
min	*mmoles/L*	*mmoles/L*	*μc/L*	*ml/min*	*ml/min*	*mmoles/L*	*mmoles/L*	*μc/L*			
0 (25 μc Sr^{85} i.v. creatinine prime; start Infusion I,* 12 ml/min)											
118–140	1.86	0.74	1.67	3.7	49	0.14	0.28	0.65	0.006	0.027	0.029
140–172	2.20	0.76	1.61	3.8	47	0.14	0.27	0.68	0.005	0.029	0.034
172–202	1.85	0.75	1.46	7.2	50	0.10	0.17	0.42	0.007	0.033	0.042
202–220	1.88	0.76	1.42	9.6	40	0.09	0.18	0.43	0.011	0.058	0.073
220 (10 g Na_2SO_4 i.v. slowly; change to Infusion II,* 3 ml/min)											
235–253	2.09	0.73	1.55	9.0	40	2.16	0.79	1.32	0.24	0.25	0.20
253–271	2.12	0.80	1.92	7.9	41	2.92	1.00	4.34	0.26	0.24	0.43
271–288	2.13	0.79	1.74	5.8	33	3.62	1.14	4.54	0.30	0.25	0.46

* Infusion I = glucose, 0.7 M; Infusion II = Na_2SO_4, 0.4 M.

the infusions. Therefore, two experiments (R2 and S) were performed without the addition of calcium to the infusions, and in three others (D, T and U), calcium was added only after several collection periods had been obtained (see Table I). One of these experiments is presented in detail in Table IV. In the first two blood samples, after starting the infusion of sulfate, plasma calcium is low due to omission of this ion from the infusion (34). Nevertheless, the strontium clearances in these periods are 45 and 42 ml per 100 ml GF. When calcium was added, plasma calcium returned to normal but radiostrontium clearance remained about the same (47 and 44 ml per 100 ml GF). Further documentation is provided in Figure 1, where the collection periods have been identified as to the presence or absence of hypocalcemia, and also of hypermagnesemia. Since magnesium salts may lead to increased calcium excretion, some increase in strontium clearance might also result. There do not appear to be any clear-cut differences between strontium clearances measured under these various conditions. Although hypocalcemia may modify the results slightly, it is clear that the major determinant of radiostrontium clearance in these experiments is sulfate excretion. These conclusions apply to clearance per 100 ml GF or uncorrected clearance, since changes in filtration rate were minor. The rate of sulfate excretion in the dogs undergoing water, glucose, or mannitol diuresis was less than 0.2 μmole per minute per

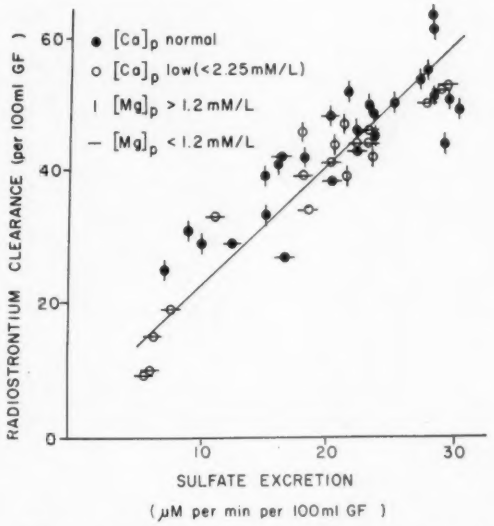

FIG. 1. RADIOSTRONTIUM CLEARANCE AS A FUNCTION OF SULFATE EXCRETION. Both parameters are expressed per 100 ml of glomerular filtrate. Radiostrontium clearance was calculated without correction for protein-binding. The relationship is approximately linear and is not significantly affected by the presence of hypocalcemia or hypermagnesemia.

100 ml of GF, and therefore cannot be shown on this graph.

Calcium and magnesium clearances were also determined in most of these experiments. The effects of sulfate infusion on the excretion of these

TABLE IV

The effect of plasma calcium on the strontiuretic action of sulfate in Dog D (8.8 kg)

Time	Plasma			Urine				C_{Ca}/GFR ratio	C_{Sr}/GFR ratio
	Ca	SO_4	Sr^{89}	Flow	GFR	Ca	Sr^{89}		
min	*mmoles/L*	*mmoles/L*	*μc/L*	*ml/min*	*ml/min*	*μmoles/min*	*μc/min*		
0 (200 μc Sr^{89} i.v. start Infusion I,* 1 ml/min)									
0–630				1.0			0.0444		
630–800	2.42	1.07		0.7			0.0186		
800 (Change to Infusion II,* 13 ml/min)									
800–830				11.6			0.506		
830–846		19.5	9.1	11.8	62	21.2	0.225		0.40
846–865	1.50	21.2	8.2	12.8	60	28.8	0.221	0.32	0.45
865–889	1.55	22.5	7.4	14.0	68	30.8	0.214	0.29	0.42
889 (Change to Infusion III,* 13 ml/min)									
889–905	1.85	22.8	7.1	14.6	60	28.9	0.201	0.25	0.47
905–928	2.28	22.9	6.8	13.4	60	35.5	0.181	0.25	0.44
970			5.7						

* Infusion I = NaCl, 0.16 M; Infusion II = Na_2SO_4, 0.090 M, K_2SO_4, 0.008 M, $MgSO_4$, 0.002 M; Infusion III = same plus $CaCl_2$, 0.005 M.

TABLE V

Radiostrontium excretion data

Dog	Per cent of injected dose remaining at start of treatment	Per cent of dose in ECF*	Per cent of dose excreted in urine during treatment	Per cent of remaining Sr excreted in urine during treatment	Treatment begun hours after initial dose
V	99.5	22	50	50	2.5
S	99.2	27	35	35	4
D	84	8	27	32	14
Q	89	3	20	22	14
R1	54	13	19	35	18
T	93	12	14	15	20
U	92	3	4	4	21
R2	29	3	4	14	41
M	66	1.6	5	8	72
E	24	0.5	1	4	168

* Extracellular fluid.

ions (as exemplified in Tables III and IV) were in accord with previously reported findings (33, 34). A comparison of simultaneous clearances of alkaline earth cations under a variety of experimental conditions is under study.

This relationship also did not appear to be affected by variations in plasma or urine pH, plasma potassium, or by the simultaneous infusion of mannitol, although a systematic study of these factors was not made.

4. The amount of radiostrontium mobilized by sulfate infusion. In Table V the ten experiments have been arranged in order of the interval between injection of isotope and commencement of the sulfate infusion. The fraction of the administered isotope remaining at the onset of therapy was determined as follows: Fecal excretion was measured directly in Dogs E, M, Q, R2 and U. In D, S and V, stools were not passed in the interval between injection of isotope and sulfate infusions. In R1 and T the stool samples were lost. Since fecal excretion of parenterally administered radiostrontium is, on the average, equal to urinary excretion in normal dogs (37), the amount of isotope collected in the urine was doubled in these five experiments in order to obtain an approximate estimate of the radiostrontium excreted prior to treatment.

The percentage of the dose in the extracellular fluid was approximated by multiplying the percentage per liter of plasma at the start of the infusion by one-fifth of the body weight.

The percentage excreted in the urine during the infusion was measured directly. The data in Table II establish that only a small fraction of this amount would have been excreted during this period without therapy.

The amount excreted in the urine during therapy exceeds the amount of isotope estimated to be in the extracellular fluid by twofold or more in five of the ten experiments. Plasma radiostrontium concentration at the end of the infusion was usually about one-half of its value at the beginning, or less. These observations establish that part of the excreted isotope was derived from sources outside of the extracellular fluid, despite the approximations involved. The efficacy of therapy may be evaluated by calculating the percentage of the remaining radiostrontium removed. As the table indicates, early therapy may remove as much as half of the body burden. Within a day, however, the remaining isotope becomes less accessible and only a small fraction can be mobilized in this manner. One week after injection, only 4 per cent of the remaining strontium was excreted during a prolonged (20 hour) infusion. These estimates are subject to some uncertainty regarding fecal excretion of isotope, and the amount of isotope remaining in the bowel.

No toxic effects were observed in these experiments, despite the large volumes infused, except when calcium or magnesium was omitted and symptoms of deficiencies of these cations developed (34).

DISCUSSION

These results indicate that sulfate is highly effective in augmenting the renal clearance of radio-

active strontium. If administered early, it significantly reduces the retention of an injected dose in dogs. In comparison with other agents which have been examined, sulfate must be given in enormous quantities. Unlike most other agents, however, sulfate can be administered in such amounts without toxic effects, provided that deficiencies of calcium, magnesium and potassium are prevented by adding these cations to the infusion (34). In the presence of normal renal function, sulfate is cleared rapidly and is therefore less likely to produce overexpansion of the extracellular fluid than is isotonic sodium chloride solution.

It remains to be determined whether these results in animals can be duplicated in man. With respect to the effects upon renal clearance, there is little reason to doubt that a similar relationship obtains, since the effects of sulfate infusion upon calcium clearance are comparable in the two species (unpublished observation). The rate of removal of radiostrontium from the extracellular fluid may well be lower in man, because the increase in extracellular fluid volume from dog to man is several fold greater than the increase in renal excretory capacity. On the other hand, the extent of skeletal deposition of radiostrontium in well nourished adults is less (38) than that in the dogs employed in this study. The net effect of these various differences between the two species cannot be assessed.

Schubert (5) has previously suggested that sulfate might be effective in promoting strontium excretion, by virtue of the lower solubility of strontium sulfate than calcium sulfate. This argument does not seem relevant because, unless carrier strontium is given, the product of strontium and sulfate concentrations in plasma or urine cannot conceivably approach the solubility product of this salt, $10^{-6.5}$ (at zero ionic strength and 25° C) (36). The concentration of strontium in normal plasma is approximately 0.3×10^{-7} M (6). Furthermore, sulfate infusion has a comparable effect upon magnesium excretion (33), despite the high solubility of magnesium sulfate. On the other hand, the reported effect of orally administered sulfate in decreasing the absorption of carrier strontium from the gut (42) is probably related to precipitation of $SrSO_4$, since strontium concentration in the gastrointestinal contents is elevated under these conditions. A single animal given carrier-free Sr^{90} and sulfate orally exhibited a 34 per cent reduction in skeletal Sr^{90} retention (43). Here the observed result may have been due to an effect of absorbed sulfate on urinary excretion of strontium, or due to impaired intestinal reabsorption of the strontium sulfate complex.

The mechanism by which parenterally administered sodium sulfate promotes strontium excretion by the kidney is not entirely clear. Several lines of evidence indicate that it is related to the formation of an electrostatic complex between strontium ions and sulfate ions, even though such a complex has apparently not been shown to exist. *1*) The effects are qualitatively and quantitatively similar to the previously reported changes in calcium and magnesium clearance during sulfate infusion. *2*) The dissociation constants of $CaSO_4$ and $MgSO_4$ are almost identical, being 0.06 M at ionic strength 0.16 (36). *3*) Sodium salts of other multivalent anions, such as ferrocyanide, also promote strontium excretion (unpublished observations).

The simplest interpretation is that strontium, when electrostatically paired with sulfate, is not reabsorbed by the renal tubules. However, it is difficult to conceive how this phenomenon can account for almost complete inhibition of radiostrontium reabsorption. Assuming that the dissociation constant of the strontium sulfate ion-pair is identical with that of calcium sulfate, only approximately one-half of urinary radiostrontium will be paired with sulfate, as calculated previously for calcium (34). Yet strontium excretion is increased many fold. Further studies of the mechanism of this effect are needed.

Protein-binding of radiostrontium was determined (44) in 3 control plasma samples and in 13 samples obtained during sulfate infusion. The ratio of ultrafiltrate strontium to plasma strontium was calculated, ignoring corrections for plasma water and the Donnan factor, which are opposite in direction and magnitude. In the three control samples, ratios of 0.68, 0.69 and 0.67 were obtained. During sulfate diuresis, the mean ratio was 0.82 ± 0.05 (SD). This decrease in protein-binding of strontium, previously found for calcium, is due to the increased ionic strength resulting from sulfate, as is demonstrated in results published elsewhere (45). The difference between the ratio for radiostrontium and that for

calcium in the same samples averaged 0.06 ± 0.05 (SD, n = 10), i.e., calcium may be slightly more strongly bound to plasma protein than is strontium. Although this difference is of borderline significance statistically, these results are in accord with previous data (6, 7, 46).

A clearance of radiostrontium of 82 ml per 100 ml GF would therefore indicate complete excretion of filtered isotope under the conditions of these experiments. The highest clearance observed was 64 ml per 100 ml GF, indicating excretion of approximately 80 per cent of the filtered isotope. No evidence for renal tubular secretion of strontium was obtained.

The minor influence of hypocalcemia upon the relationship depicted in Figure 1 seems surprising, since it is known that administration of calcium leads to augmented radiostrontium excretion. However, the calcium clearance in a sulfate-loaded animal, in contradistinction to that of a normal animal, is independent of the plasma calcium concentration (34). Thus, calcium administration leads to an increase in calcium *excretion*, but under these conditions it fails to influence calcium *clearance*. Radiostrontium clearance is also unaffected (see Table IV).

It does not necessarily follow that the efficacy of therapy is unaffected by the level of plasma calcium. Even though radiostrontium *clearance* remains constant, the rate of radiostrontium *excretion* during sulfate infusion may be related to the plasma calcium. This would be the case, for example, if radiostrontium were mobilized from skeletal deposits more rapidly in the presence of hypocalcemia. Attempts to settle this point were unsuccessful, because of the variability between dogs and the unpredictable rate of decline of plasma radiostrontium in any given dog. In practice, the addition of calcium to the infusion is necessary in order to prevent symptomatic hypocalcemia.

This mode of therapy of radiostrontium poisoning, therefore, does not fall in the category of "decalcification therapy" (3). It is possible that prolonged administration of sulfate in smaller dosage without calcium might accomplish a degree of skeletal demineralization which would prove effective in mobilizing long-standing skeletal deposits of radiostrontium or other radioelements. A preliminary study employing oral methionine or thiosulfate to augment urinary sulfate excretion, has been reported in abstract form (1). Calcium intake in this study was not reduced sufficiently to permit a large negative balance to occur. In theory, however, any degree of skeletal decalcification can be induced by prolonged sulfate administration, as for example by intravenous injections of hypertonic sodium sulfate several times daily. The question is whether skeletal radiostrontium deposits will be mobilized early or late in such a procedure; in other words, whether the ratio of radiostrontium to calcium in the urine will pass through a peak value soon after instituting therapy or remain at a low value indefinitely. This problem remains to be elucidated.

The properties of an ideal agent for removing radiostrontium have been discussed by Schubert (5). It was assumed that the fraction of circulating strontium bound by the agent was a measure of its efficiency. More practical criteria should include two properties; the maximum strontium clearance attainable and the rate of removal of radiostrontium from bone. With respect to the former criterion, sulfate appears to be unexcelled; however, it is distinctly limited in its ability to mobilize skeletal deposits. The combined use of sulfate and skeletal dimineralizing agents may prove to be an effective form of therapy for radiostrontium poisoning.

SUMMARY

Carrier-free isotopes of strontium, Sr^{85} and Sr^{89}, were injected intravenously into dogs. At intervals of 2.5 to 168 hours later, solutions containing sulfate were infused for 2 to 20 hours. The rate of urinary radiostrontium excretion immediately increased 6- to 40-fold. Radiostrontium clearance per 100 ml glomerular filtrate increased linearly with sulfate excretion. The fraction of filtered strontium excreted reached 80 per cent as compared with a normal value of less than 10 per cent. During sulfate infusion, 22 to 50 per cent of the estimated amount of isotope remaining was excreted in the urine, provided that therapy was instituted within 18 hours of injection. When therapy was delayed, less than 15 per cent of the remaining radiostrontium was removed. In half of the experiments, the amount of isotope excreted in the urine during sulfate infusion was more than

twice the amount estimated to be in the extracellular fluid, indicating that deposits of radiostrontium were mobilized from the skeleton and/or the tissues.

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STUDIES ON THE EFFECTS OF HUMAN GROWTH HORMONE IN PREMATURE INFANTS*

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The role of pituitary growth hormone during fetal and neonatal life remains uncertain even though it has been demonstrated repeatedly that hypophysectomy of the juvenile animal severely retards or arrests growth. The claim has been made that administration of growth hormone to normal pregnant rats stimulates growth of the fetus (2–5), but the evidence presented is inconclusive. In man, the macrosomia observed in infants born to prediabetic or diabetic mothers has been attributed by some to an excess of maternal circulating growth hormone (4, 6–9), thus implying transplacental passage of this growth-promoting substance and response of the fetal organism to exogenous hormone. Conversely, the normal birth weight of infants born to women with active acromegaly (10–12) and the relatively unimpaired growth of apituitary fetuses do not seem to support this hypothesis (13–18).

The biological effectiveness in man of primate growth hormone preparations has been clearly established (1, 19), as has the lack of effectiveness of bovine, ovine or porcine growth hormone, providing an example of species specificity for a protein hormone (20). Accordingly, conclusions drawn from isolated reports of the effects of nonprimate growth hormone in infants may be questioned. The effects in the immature infant of the administration of a purified preparation of human growth hormone have been assessed in an attempt to elucidate the relationship of pituitary growth hormone to fetal and neonatal body growth.

MATERIALS AND METHODS

Five healthy premature infants weighing between 1,500 and 1,600 g at approximately 2 weeks of age and growing at a relatively steady rate were selected at random. The infants were fed a formula consisting of 12 g of Alacta [1] and 7 g of glucose per 100 ml water, which provided 0.77 calorie per ml. Feedings were administered every 3 hours through a polyethylene nasogastric tube until such time as the entire formula was taken adequately by nipple. Some infants at the end of the period of investigation were fed an evaporated milk formula consisting of 1 part of evaporated milk, 2 parts of water and 5 per cent cane sugar, which contained 0.66 calorie per ml. The caloric intake was kept constant at 120 cal-

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[1] Alacta (Mead Johnson & Co.), a powdered milk preparation, contains 4.3 calories per g, 33.0 per cent protein, 12.0 per cent fat and 46.5 per cent carbohydrate.

TABLE I

Comparison of the treated infants and the dose schedule of hormone administration

Subject	Sex	Race	Birth wt	On day 0*		Growth hormone in mg on Day									Total
				Age	Wt	0	1	2	3	4	5	6	7	8	
			g	days	g										mg
Fel.	M	W	1,389	40	1,980	1									1
McM.	F	W	1,380	32	1,910	1		1		1		1		1	5
Hea.	F	W	1,420	31	1,950	2	2	2	2	2					10
Pig.	F	W	1,400	29	1,760	5	5	5							15
Mor. B	M	W	1,474	22	1,910	10	10	10							30
Bar.	F	W	1,417	38	2,080	ACTH, 15 U q.d. × 13 days Growth hormone, 10 mg q.d. × 4 days									40

* Day 0 corresponds to the first day of growth hormone administration, except for Bar. for whom it is the first day of ACTH administration. Growth hormone was injected into Bar. on Days 10 to 13 inclusive.

TABLE II

Comparison of infants in the control group

Subject	Sex	Race	Birth wt	On day 0*	
				Age	Wt
			g	*days*	*g*
Mor. A	M	W	1,620	22	1,960
Bra.	F	W	1,474	19	1,950
Cam.	M	W	1,701	20	1,820
Stu.	M	W	1,559	23	1,920
Kno.	M	W	1,588	23	2,000
Ros.	M	W	1,588	24	2,010
Gaf.	M	W	1,332	33	1,910

* Day 0 is a hypothetical day when growth hormone would have been administered had these subjects been treated.

ories per kg of body weight per day by adjusting the amounts of formula every second day.

Human growth hormone was prepared by Dr. Maurice Raben from pituitary glands collected at autopsy and stored in acetone (1, 21). The powdered growth hormone was diluted in normal saline and the pH adjusted to 3.5 by addition of dilute hydrochloric acid. It was injected intramuscularly as a clear solution. No local or systemic reactions were encountered in any of the treated subjects.

Each infant was followed for a minimum of 3 weeks from the time he began to grow at a steady rate. The periods of study selected were the week before, the week during, and the week after growth hormone administration, each infant serving as his own control (Table I). In addition, a comparable group of 7 untreated infants was evaluated during the same time interval, and served as an additional control group (Table II).

The effects of growth hormone were studied by the following parameters.

A. Growth-promoting effects. Growth was estimated by: *a*) daily weight, *b*) head circumference measured in triplicate 6 days a week, and *c*) bi-weekly estimation of fibula length from X-ray films [2] exposed at a constant target-film distance of 38 inches, according to the method of Day and Silverman (22). The fibula method for assessing linear growth was used because of its sensitivity and reproducibility and because of the inaccuracies inherent in measurements of body length in this age group. It reflects accurately general skeletal growth and can detect a significant increment in growth in infants in as short a period of time as 2.5 days (22). During exposure, care was taken to protect the gonads from radiation.

B. Metabolic effects. Determinations of the concentration of blood urea nitrogen (23, 24), blood sugar (23, 25), and serum phosphorus (23, 26) were obtained thrice weekly on venous blood collected according to a scalp vein technique previously described (27). The latter made it possible to obtain readily small serial samples of venous blood required for selected chemical determinations.

In 2 infants of comparable weight and age, plasma free fatty acids (FFA) (28) were determined in the fasting

[2] Before obtaining roentgenograms of the fibula, the dose of radiation involved was carefully considered by the Pediatric X-ray Department. Dr. David H. Baker estimated the exposure to radiation as follows: "Radiographs were obtained using a Picker 500 ma machine equipped with a Picker double-diaphragm localizer. The gonads were shielded. The factors used were: 33 kv, ⅓ second, 400 ma at 38 inches. The calculated skin dose was 14 milliroentgens. The estimated fibula dose was less than 50 per cent of the skin dose. Radiation to the gonad and whole body was negligible. For comparison with the above, the average skin dose for a typical posterior-anterior chest film obtained with a standard X-ray machine is approximately 100 milliroentgens."

TABLE III

Weekly increments in weight, fibular length and head circumference during the study period

		First week			Second week			Third week			
		Head circumference	Wt	Fibular length	Head circumference	Wt	Fibular length	Head circumference	Wt	Fibular length	Growth hormone
Control subjects		*cm/wk*	*g/wk*	*mm/wk*	*cm/wk*	*g/wk*	*mm/wk*	*cm/wk*	*g/wk*	*mm/wk*	*mg*
Mor. A		1.27	200	1.74	1.50	340	1.27	1.10	170	1.73	0
Bra.		1.10	250	1.33	0.77	120	1.65				0
Cam.		1.23	180	1.10	1.23	290	1.12	1.07	130	2.03	0
Sto.		1.14	280	1.43	1.01	190	1.97	0.95	150	1.33	0
Kno.		1.43	290	1.85	0.64	230	0.60	0.90	290	1.80	0
Ros.		1.16	340	1.93	0.84	200	1.27	0.56	110	1.20	0
Gaf.		0.80	250	1.92	1.00	190	1.33	0.36	140	0.70	0
	Mean	1.16	255.71	1.61	1.00	222.86	1.32	0.82	165.0	1.47	
	SE	0.07	20.57	0.12	0.11	27.40	0.16	0.12	26.30	0.20	
Treated subjects		*Week prior to growth hormone administration*			*Week of growth hormone administration*			*Week following growth hormone administration*			
Fel.		1.24	260	1.50	0.80	270	0.95	0.56	100	1.20	1.0
McM.		1.17	210	1.10	1.0	160	1.40	0.90	170	1.60	5.0
Hea.		1.30	210	1.70	0.77	300	1.40	1.09	190	1.10	10.0
Pig.		0.97	190	1.70	0.87	280	1.50	0.77	180	1.60	15.0
Mor. B		1.30	260	1.05	1.00	290	2.18	0.84	150	1.50	30.0
	Mean	1.20	226.0	1.41	0.89	260.0	1.49	0.83	158.0	1.40	
	SE	0.12	14.35	0.14	0.05	25.50	0.20	0.09	15.94	0.10	

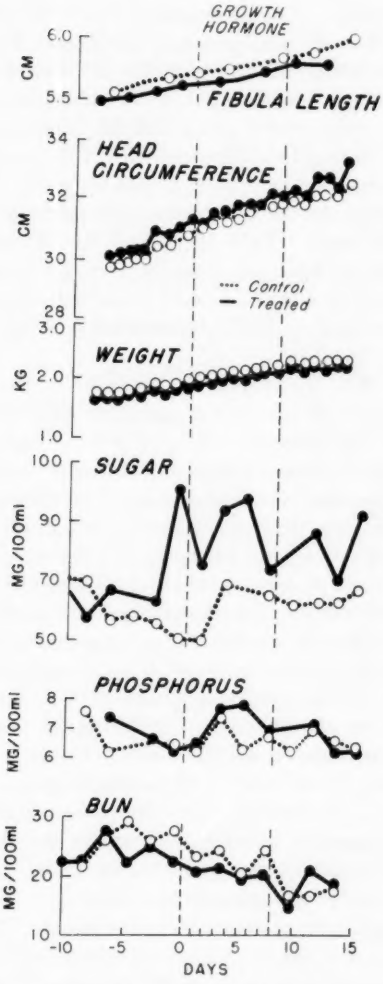

FIG. 1. A COMPARISON OF THE MEAN VALUES FOR VARIOUS PARAMETERS OF GROWTH HORMONE ACTIVITY IN PREMATURE INFANTS TREATED WITH HUMAN GROWTH HORMONE (DOTS AND SOLID LINES) AND IN CONTROL SUBJECTS (OPEN CIRCLES AND INTERRUPTED LINES). Human growth hormone was administered during the 8 day period between the vertical broken lines (see Table I for schedule of dosage).

state over a 4 hour period beginning at 8 a.m. In order to compare changes in the concentration of FFA elicited by fasting alone, and by fasting with the administration of growth hormone on the following day, 1.25 mg of human growth hormone was injected intravenously over a 2 to 3 minute period after a 3 hour fast; the plasma FFA content was measured prior to injection and during the succeeding 4 hour period.

In addition, in 5 premature infants the concentration of "growth hormone" in serum was determined by the immunoassay method described by Read (29). Studies indicating a high degree of immunologic homogeneity of the Raben preparation of human growth hormone and of specificity of the rabbit anti-human growth hormone serum have been reported recently from our laboratory (30).

C. ACTH and growth hormone. One subject received sufficient ACTH to inhibit growth, at which time growth hormone was added in an attempt to evaluate its effect on the growth-suppressing action of ACTH. After a period of 4 days, both preparations were discontinued and the infant's response was observed (Table I).

RESULTS

Tables I and II compare the two groups of infants investigated. Table I shows the dosage and pattern of growth hormone administration in the treated subjects.

A. Growth-promoting effects. In Table III and Figure 1 are shown the mean values for measurements obtained in the control and treated groups of infants, expressed in weekly increments. Both groups were entirely comparable by all indices

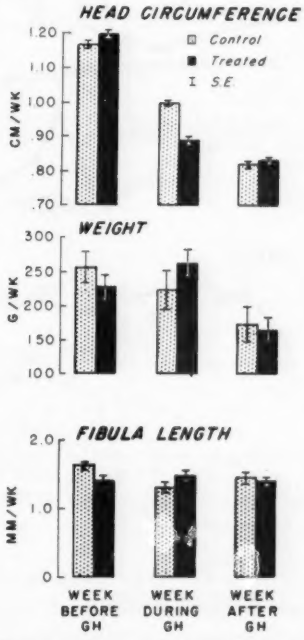

FIG. 2. THE EFFECT OF HUMAN GROWTH HORMONE ON INDICES OF GROWTH IN THE PREMATURE INFANT. The stippled bars denote mean weekly increments for the control subjects and the solid bars for the treated subjects. The gradient represents ± standard error of the mean.

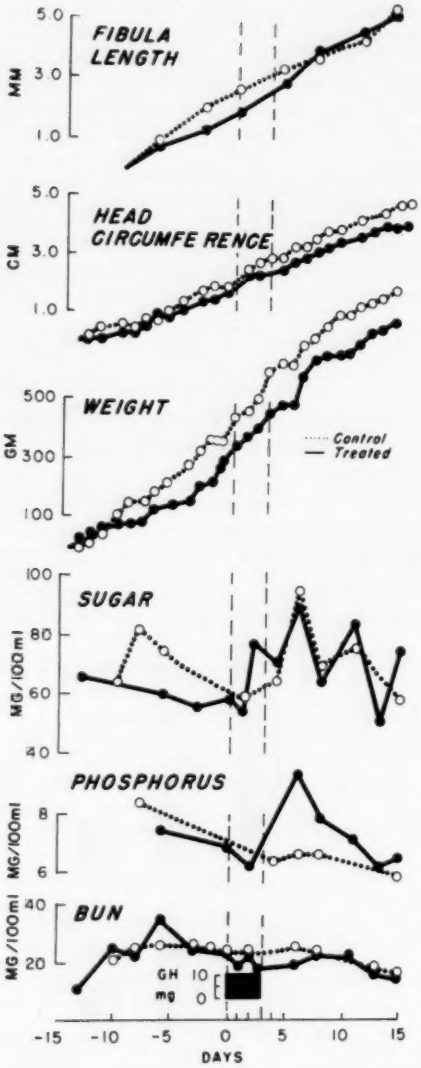

FIG. 3. THE EFFECT OF HUMAN GROWTH HORMONE IN MOR. B (DOTS AND SOLID LINES) IN CONTRAST TO HIS UNTREATED IDENTICAL TWIN, MOR. A (OPEN CIRCLES AND INTERRUPTED LINE). Measurements are expressed in cumulative increments.

studied (Figure 2). The mean differences between the two groups for head circumference, weight and increments in fibular length were not significant by Student's *t* test (31).

Mor. A and Mor. B, identical twins (monozygosity suggested by the presence of a single placenta and identical blood group antigens), were also studied. The increment in fibular length for twin Mor. B who received the largest dose of growth hormone (10 mg a day for 3 days) was significantly greater ($p < 0.001$) than the mean value of the control group and the increment observed during the control period. This trend was not seen when twin Mor. B was compared to the control group for increments in weight and head circumference. Twin Mor. A on the other hand, showed no departure from the rest of the control group. In Figure 3 both twins are compared with respect to head circumference, weight and fibular length.

B. Metabolic effects. In the subjects who were given growth hormone a significant rise in blood sugar was observed ($0.05 > p > 0.02$) from pretreatment values (Figure 1). In no instance was hypoglycemia encountered. In most infants studied, in both the control and treated groups, the level of blood urea nitrogen (BUN) was somewhat elevated, but the expected fall with advancing age was not significantly influenced by administration of growth hormone. Nevertheless, there was a tendency with the larger doses of growth hormone for the concentration of BUN to fall and of serum phosphorus to rise during the period of growth hormone administration (Figure 1) to a greater extent than in the control group of infants. This trend was also observed when Mor. A was compared with his monozygous twin Mor. B with respect to the concentration of BUN, blood sugar and serum phosphorus (Figure 3).

The results of determination of plasma FFA are shown in Table IV. The concentration of plasma FFA rose appreciably in the two infants studied, but no increase above the fasting values was observed following infusion with growth hormone.

The concentration of serum "growth hormone" in five premature infants varied between 230 and 480 mμg per ml, values within the range found in older infants and children by Read (29) and in our laboratory.

C. ACTH and growth hormone. In the subject who received ACTH, growth inhibition was achieved, in agreement with previous studies (32, 33). The growth-suppressing action of ACTH was not altered by simultaneous administration of growth hormone, and the rebound which occurred following withdrawal of ACTH and growth hormone (Figure 4) was not greater than that ob-

TABLE IV

The effect of fasting and of human growth hormone on the concentration of plasma free fatty acids

Subject	Plasma free fatty acids (μEq/L)					
	Control (Day 1)			Growth hormone (Day 2)		
	0 hr*	2 hrs	4 hrs	0 hr*	2 hrs	4 hrs
1	200	240	420	140	400	350
2	110	370	820	370	650	750

* 0 Hour corresponds to the time of growth hormone administration (1.25 mg i.v.) after a 2 to 3 hour fast. The subjects were tested on successive days.

served (32) in premature infants when ACTH was discontinued.

DISCUSSION

The data indicate that the immature human being is relatively unresponsive to the particular preparation as well as to the dosages of human growth hormone employed in these experiments. In contrast, this lot of Raben human growth hormone has been shown to exhibit highly potent biological activity when assayed in the hypophysectomized rat (1, 21) and to induce significant linear growth in hypopituitary dwarfs (1, 19, 34, 35), effects similar to those obtained with primate growth hormone prepared by Li [see Hutchings and co-workers (36)]. These, or comparable preparations of human growth hormone, have also shown significant biological activity in normal adult subjects and in persons with non-endocrine disorders (1, 19, 37–39). In some of the latter studies (37, 38), the dose of hormone and the schedule of administration were similar to those used in the present experiments. Although biological changes, including a positive nitrogen balance, were clearly demonstrable in adult subjects, in three children (39), and in one 16 month old infant (40), the effect of growth hormone was more pronounced in hypopituitary dwarfs (1, 19). On the other hand, a relative lack of responsiveness (as calculated on the basis of body weight or of surface area) was exhibited by the premature infant to comparably large doses of human growth hormone. Only in Mor. B, the subject who received the largest dose of growth hormone, was a true growth-promoting effect obtained. It may be argued that balance studies would have provided a more sensitive index of growth or of a protein anabolic response. However, serial measurements of the length of the fibula make possible the early and accurate detection of growth, and this method also assesses directly the stimulating effect of growth hormone on osteogenesis. In addition, the results obtained from the other parameters selected in the present study to detect biological activity of exogenous growth hormone suggest that determinations of the output of nitrogen, phosphorus, and potassium in relation to their intake would not have provided a greater degree of sensitivity or precision.

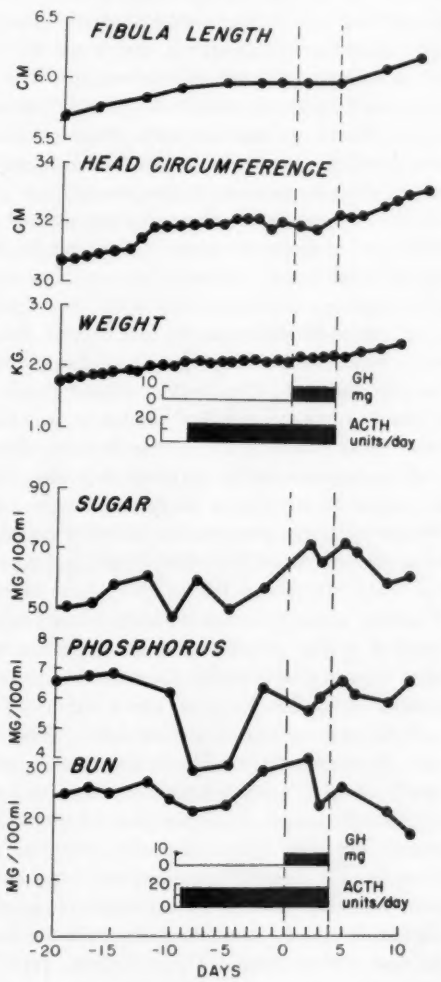

FIG. 4. THE EFFECT OF HUMAN GROWTH HORMONE ON THE GROWTH-SUPPRESSING ACTION OF ACTH IN A PREMATURE INFANT.

When growth hormone is administered in an acid medium, its diabetogenic effect is diminished while its growth-promoting activity remains unaffected (1, 41). The Raben preparation is virtually nondiabetogenic in hypopituitary dwarfs, but Beck, McGarry, Dyrenfurth and Venning (19) noted decreased glucose tolerance in one subject who received large doses of hormone for 20 days. Young (42) has postulated that a capacity for linear growth seems to protect the young animal against the diabetogenic activity of growth hormone. The infants in this study responded to growth hormone with a significant rise in blood sugar content. It is noteworthy that the dose of growth hormone administered was large in relation to body weight and surface area.

Whereas hypophysectomy leads to a fall in serum phosphorus, growth hormone promotes phosphorus retention and an increased concentration of serum phosphorus and alkaline phosphatase (1, 13, 43). In contrast to the preparation used in the British cooperative study (37), the Raben preparation of human growth hormone has uniformly produced a gradual rise in the concentration of serum phosphorus to prepubertal levels when administered to hypopituitary dwarfs and to normal adults (1, 19). In the treated group of premature infants a similar tendency was observed. Parenthetically, in the infant whose growth was temporarily arrested following administration of ACTH, a sharp fall in the concentration of serum phosphorus occurred initially.

Recently Raben and Hollenberg (44) and Engel, Bergenstal, Nixon and Patten (45) have shown that human growth hormone, when administered to normal adult subjects and to hypopituitary dwarfs, causes a substantial increase in the concentration of plasma FFA, an effect attributed to the adipokinetic action of the hormone. The response of plasma fatty acids in the fasting state to small doses of growth hormone seems to be a highly sensitive and rapid method for detecting a growth hormone effect (1). It is of interest that exogenous growth hormone did not induce such a response in the two premature infants studied, although the concentration of plasma fatty acids rose with fasting. These findings provide additional evidence of the relative unresponsiveness of the immature human subject to administered growth hormone.

The role of growth hormone in the fetus and neonate is poorly understood. The resistance of the immature human being to exogenous growth hormone could be attributable to the fact that growth hormone at this stage of the life cycle does not play an essential part in embryonic, fetal, and early postnatal growth, or that its action on this process is primarily a permissive one. On the other hand, it is also possible that the fetus and neonate are already stimulated maximally by endogenous sources of growth hormone.

Hypophysectomy of either the mother or fetus has been reported to have no effect, or only a slight one, upon fetal growth in the chick, mouse, rat or rabbit (12, 46–50). Smith (51) has shown that the offspring of monkeys hypophysectomized during pregnancy were of normal sitting height at birth and had a birth weight within the normal range, although lower than that of control animals. Lack of a fetal hypophysis, occurring spontaneously or following experimental ablation, in the chick, mouse, rat [3] and rabbit either had no effect on body growth or was associated with near normal growth (12, 46–49). Jost and Picon (47) concluded from the results of their experiments and those of other experimental embryologists: "So far, . . . no hormone is known which is essential to the foetus for the performance of its share in achieving its own growth."

In placental mammals direct evidence is lacking that growth hormone is transferred across the placenta. Yet, in man as well as in the chick, mouse, rat and rabbit, absence of the fetal hypophysis does not seem to influence, to any degree, embryonic and fetal growth. Apituitary and acephalic human fetuses in general have not shown appreciable deviations from the norm in length or weight at birth when these measurements are corrected for the cerebral defect (15–18, 52). An

[3] Wells (49), with Heggestad, found that rat fetuses decapitated 50 hours before term weighed less than litter mates decapitated at delivery. In contrast to other workers, they interpreted their experiments as indicating that growth hormone may affect the weight of the fetus. However, it is questionable whether the unoperated litter mates may be considered a proper control animal. In view of the effects of operative manipulation on fetal animals we have chosen to give most weight to those experiments in which a negligible influence of fetal hypophysectomy on growth was demonstrated.

infant born of a mother hypophysectomized during the twenty-fifth week of pregnancy for carcinoma of the breast was of normal size when delivered at 35 weeks (53). Similarly, infants born of mothers with active acromegaly have not been excessively large (10–12).

The normal birth weight and growth during infancy of idiopathic hypopituitary dwarfs seem to support further the limited influence of growth hormone as a growth-promoting agent during gestation and infancy (14, 54). It has been shown that rats hypophysectomized as early as 6 days of age continue to grow at approximately half the rate of the unoperated control group until about 30 days after birth, after which time growth stops (55, 56). In contrast, cessation of body growth occurs rather abruptly following the removal of the rat's hypophysis at 28 days. Mice affected with hereditary anterior pituitary dwarfism exhibit a steady rate of growth for the first week or so of life, although degenerative changes in the eosinophilic cells of the anterior hypophysis are detectable microscopically before birth (57). These observations would seem to indicate an appreciable capacity for growth of placental mammals in early life independent of the anterior hypophysis.

The detection of growth hormone in the plasma of the premature infant indicates that at this age the anterior hypophysis is capable of synthesizing and secreting sufficient growth hormone to maintain a concentration in the circulation at least equal to that found in children. Indeed, Smith and Dortzbach (58) have demonstrated that early in fetal life the hypophysis of the fetal pig has a specific growth-promoting effect when implanted into the hypophysectomized rat, and the activity once detected increased with advancing fetal age. Gershberg (59) has shown that the concentration of growth hormone in human pituitary glands, as determined by bioassay, varies little with age.

It is common knowledge that growth hormone has a general anabolic action and effects an increase in the mass of most body tissues. A considerable body of data, recently reviewed (1, 14, 60–62), provides substantial evidence of the influence of growth hormone on carbohydrate, fat, protein and mineral metabolism. Nonetheless, the primary action of growth hormone is still obscure. Recently, there has been renewed interest in the effect of growth hormone on the mobilization and oxidation of fat and the relationship of this effect to its anabolic action. Greenbaum (63, 64) has shown, from carcass analyses of normal and growth hormone-treated rats on a restricted diet, that protein synthesis ceases when available fat reserves for catabolism have been exhausted. Evidence adduced from an accumulating body of data (1) suggests, but has not established, that an intrinsic effect of growth hormone is to promote the catabolism and utilization of fat which, in the presence of an adequate supply of dietary amino acids, leads to protein deposition and diminished protein oxidation. It is of interest that obesity is exceedingly rare in acromegaly.

In the light of the action of growth hormone on adipose tissue it is noteworthy that the premature infant has little subcutaneous fat. Fehling in 1877 (65) and Wilmer in 1940 (66, 67) demonstrated that the fat content of the fetus increases rapidly during the third trimester of gestation. In a comprehensive study of body composition by Widdowson and Spray (68), most of the increase in fat stores was found to occur between the end of the eighth month and term. Carcass analysis of a 1,500 g premature infant showed only 3.5 per cent fat as compared with analyses of a 2,500 and a 3,500 g infant which yielded 7.6 per cent and 16.2 per cent fat, respectively. It is necessary to consider the possibility that the meager labile fat stores characteristic of the premature infant may play a role in the relative unresponsiveness of the premature infant to the administration of growth hormone.

On the other hand, the premature infant on a diet providing adequate calories and protein often grows at a near maximal rate which may, at least in part, be independent of endogenous growth hormone. Furthermore, the premature infant when fasted shows a rise in the concentration of plasma FFA which indicates an ability to mobilize fat. Hence, it is curious that no additional increase in the concentration of FFA was obtained following the injection of a large dose of growth hormone. In addition, growth hormone failed to counteract the growth-suppressing action of ACTH in the subject who received both hormones concurrently. This finding contrasts with the observation of Selye in the rat (69) and the positive nitrogen balance obtained with human growth

hormone in an adult with Cushing's syndrome (39).

The data obtained in this study indicate that the premature infant is relatively unresponsive to the growth-promoting and metabolic effects of exogenous growth hormone. It does not, however, establish whether growth in the newborn and neonatal period is dependent on growth hormone as it is at later ages or whether, as appears to be the case in the fetus, it is essentially independent of circulating growth hormone and related to an intrinsic capacity for growth. It would seem from the available information that the young infant is in a transitional state, in which growth hormone assumes a progressively more significant function as a regulator of body growth. Although in the present study the period of growth hormone administration was short, the limited effect in our subjects provides additional evidence (70) against the notion that the macrosomia observed in infants born to prediabetic and diabetic mothers is induced by maternal growth hormone transferred across the placenta.

SUMMARY

Human growth hormone was administered intramuscularly to six premature infants and the effects assessed by comparison with the pre- and posttreatment periods and with a control group of seven subjects.

1. A definite increase in the concentration of blood sugar was elicited; the concentration of serum inorganic phosphorus tended to increase and the concentration of blood urea nitrogen to decrease.

2. A significant increment in head circumference and weight was not obtained, nor was an increase in the rate of growth of the fibula detected, except in one subject who received the largest amount of growth hormone (10 mg per day for three days).

3. Growth hormone injected intravenously failed to augment the increased concentration of plasma free fatty acids obtained by fasting alone.

4. Suppression of growth induced with ACTH was not affected by the concurrent administration of growth hormone.

5. Growth hormone was detected in the plasma of the premature infant.

It is concluded that the premature infant is relatively unresponsive to the action of exogenous growth hormone. This contention is examined in the light of results of experiments in placental mammals and the findings in fetal and postnatal hypopituitarism in man.

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METABOLISM OF 7-H^3-EPINEPHRINE-d-BITARTRATE IN NORMAL YOUNG MEN

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Because of the numerous and important physiological and biochemical actions of epinephrine, the inactivation and metabolism of this and other catechol amines have been the subject of considerable interest, especially in recent years. In 1951 Schayer demonstrated in the urine of rats the presence of four metabolic products of injected C^{14}-epinephrine, but these remained unidentified (1). As recently as 1957 the major pathways and products of catechol amine metabolism were unknown, although it was assumed that the primary route of disposition was mainly by way of oxidative deamination (2, 3).

In 1957 Armstrong, McMillan and Shaw identified 3-methoxy-4-hydroxymandelic acid as a major metabolite of epinephrine and norepinephrine (4). Considering the possible routes of metabolism of the catechol amines in the light of this report, Axelrod sought and demonstrated a new pathway of epinephrine metabolism by 3-O-methylation (5). He showed that 3-O-methyl epinephrine (metanephrine) was formed *in vitro* when a nonparticulate fraction of rat liver was incubated with epinephrine and S-adenosyl-methionine (5), and that metanephrine was excreted in the urine of rats, both under normal conditions and after the intraperitoneal administration of *l*-epinephrine bitartrate (5).

The magnitude of the O-methylation pathway in rats was estimated by Axelrod, Inscoe, Senoh and Witkop. They concluded that about 70 per cent of epinephrine was O-methylated (6). In 1958 a preliminary report was published by LaBrosse, Axelrod and Kety, which indicated that O-methylation was also the principal route of metabolism of epinephrine in man (7).

Earlier studies on epinephrine metabolism in man (7–10) were necessarily limited by insufficient knowledge of the nature of the metabolites, lack of adequate methods for their quantification or incomplete accountability of the excretion of the infused radioactivity, and accurate evaluation of the alternate pathways was not possible.

The recent identification of an additional metabolite of epinephrine, 3-methoxy-4-hydroxyphenylglycol, by Axelrod, Kopin and Mann (11), the use or development of improved methods for the separation and estimation of this and other metabolites, and the advantages associated with the use of tritium-labeled epinephrine of high specific activity have warranted a further examination of the metabolites of epinephrine in man.

METHODS

Materials. *d,l*-Epinephrine-7-H^3-d-bitartrate (specific activity 5.0 mc per mg) was used.[1] When developed in both the phenol : 0.01 N HCl–SO_2 (80 : 20) and the *n*-butanol : acetic acid : water (4 : 1 : 1) systems, chromatograms of this tritium-labeled epinephrine showed one peak of radioactivity corresponding to authentic epinephrine-d-bitartrate. The tritium label (7-H^3) of epinephrine did not exchange with the hydrogen in the water of aqueous solutions of this compound when stored in 2 per cent sodium metabisulfite solution for several months at 4° to 8° C.

Administration of epinephrine. *d,l*-Epinephrine-7-H^3-d-bitartrate, alone or with added unlabeled *l*-epinephrine-d-bitartrate, was infused intravenously at rates of approximately 0.3 μg of *l*-epinephrine base per kg per minute or 0.18 μg per kg per minute for 30-minute periods to 12 normal young men, 18 to 22 years of age, weighing from 59.4 to 79.1 kg. The total dose ranged from 311 to 1,101 μg of *l*-epinephrine (free base), and specific activity ranged from 342 to 971 μc per mg. The details of its administration and the results of physiological (12) and psychiatric studies (13) on these subjects are reported elsewhere.

Collection of urine and determination of total radioactivity. Urine was collected without preservative for

[1] Prepared by the New England Nuclear Corporation by the reduction of adrenalone with lithium aluminum tritide; labile tritium was removed, and the *d,l*-epinephrine-7-H^3-d-bitartrate was obtained as a grayish-white powder.

54 hours following the infusion and was promptly refrigerated until analyzed or stored in the deepfreeze if the analysis was delayed. The total radioactivity in each of the urine specimens was determined by a modification of the method of Okita, Spratt and Leroy (14) by adding 0.1 ml of urine to a mixture of 4 ml of ethanol and 10 ml of 0.4 per cent 2,4-diphenyloxazole and 0.01 per cent *β-bis*(2-phenyloxazole) benzene in toluene ("ethanol-fluor"). Radioactivity was assayed in a liquid scintillation spectrometer. Duplicate portions of urine were used throughout in these studies. With all samples, correction for quenching was made by counting before and after the addition of tritium internal standard, and correction for partition was made by using authentic compounds in the respective extraction procedure. Since difficulties are usually encountered in obtaining complete urine collections, the values for total urinary radioactivity which were obtained are probably underestimates.

Estimation of metanephrine. Free metanephrine was determined in duplicate 2 ml portions of the urine specimens, by adjustment to pH 10 with 6 N NaOH and 0.2 ml of pH 10 borate buffer (0.8 M), followed by extraction with 10 ml of isoamyl alcohol. After centrifugation, a 4 ml portion of the isoamyl alcohol phase was transferred to vials containing the ethanol-fluor solution and the radioactivity was assayed in the scintillation spectrometer.

Metanephrine glucuronide was determined by adding 0.2 ml of pH 6.5 acetate buffer containing 1,400 U of bacterial β-glucuronidase (Sigma) and 6 drops (0.3 ml) of chloroform to 2-ml portions of the urine. The urine was incubated for 3 hours at 37° C. After the incubation, 1.8 ml of borate buffer (pH 10, 0.8 M) was added and the solutions were extracted with isoamyl alcohol. Four-ml portions of the isoamyl alcohol extract were counted for radioactivity as above. This isoamyl extract obtained following glucuronidase hydrolysis contains the initial free metanephrine and metanephrine released by the enzyme. Therefore, radioactivity in the metanephrine glucuronide was calculated by subtracting that in the free metanephrine from that obtained after β-glucuronidase hydrolysis.

Metanephrine sulfate was determined by acid hydrolysis followed by extraction at pH 10 as follows: Six N HCl, 0.4 ml, was added to 2-ml portions of the urine and the solutions were kept at 37° C for 48 hours in a water bath. The hydrolysates were adjusted to pH 10 with 0.4 ml of 6 N NaOH and 0.4 ml of pH 10 borate buffer (0.8 M). After extraction with 10 ml of isoamyl alcohol and centrifugation, two 4-ml portions of the organic phase were taken for determination of the radioactivity. Under the conditions of acid hydrolysis, a negligible amount of the metanephrine is freed from the glucuronide; therefore, the radioactivity which is extracted at pH 10 after the acid hydrolysis arises from free metanephrine and hydrolyzed metanephrine sulfate. Subtraction of the "free" metanephrine gives the value for the metanephrine sulfate. When corrections were made for the aliquots which were used, authentic metanephrine carried through these procedures gave recoveries of 80, 76, and 70 per cent for the procedures for estimation of free metanephrine, metanephrine glucuronide, and metanephrine sulfate, respectively. These recoveries were used in calculating the amount of radioactivity represented by each of the metabolites.

Estimation of 3-methoxy-4-hydroxymandelic acid (VMA). VMA was estimated in 2-ml portions of urine by adjusting the urine to pH 1 with HCl and extracting with 20 ml of isoamyl alcohol. Fifteen ml of the isoamyl alcohol phase was shaken with 3 ml of 5 per cent sodium bicarbonate. Two ml of the sodium bicarbonate extract was adjusted to pH 1 with HCl and extracted with 10 ml of isoamyl alcohol. Radioactivity in two 4-ml portions of the isoamyl alcohol was determined as above. Authentic VMA gave a recovery of 80 per cent when carried through this procedure. Corrections for quenching, aliquoting and partition were made as above for the metanephrine metabolites.

As a check on the extraction procedure, VMA was also determined by chromatography on silica gel. Silica gel columns were prepared by adding 2.5 ml of 0.5 N H_2SO_4 to 4 g of dry silica gel. This acidified silica gel, a free-flowing powder, was suspended in chloroform and poured into a column 1 cm in diameter to a height of 5 cm. A 1 ml portion of urine was acidified with 0.1 ml of 10 N H_2SO_4, mixed with 2 g of dry silica gel and added to the column. The column was topped with a snug-fitting filter paper disc and washed with 20 ml of chloroform. The VMA was eluted with 80 ml of 5 per cent *n*-butanol in chloroform. Recoveries of authentic H^3-VMA added to urine were 90 to 95 per cent. Determination of VMA by the extraction procedure and by chromatography on silica gel showed good agreement (respectively, 41.2 and 40.6 per cent average of the radioactivity in the urine).

The specificity of these procedures is shown by the presence of one peak of radioactivity which corresponds to the position of authentic compounds on paper chromatograms of these fractions (see Figures 1A and 1B).

Estimation of free epinephrine in urine. Free epinephrine in the urine was determined by chromatography of 10-ml portions of the urine on alumina columns (15). Dry nonalkaline alumina (Woelm) was poured into columns 1 cm in diameter to a height of 4 cm, and washed with 10 ml of 0.5 M borate buffer, pH 8.3, followed by 20 ml of glass-distilled water. One ml of 1 per cent sodium ethylenediamine tetraacetic acid (EDTA), 1 mg of nonradioactive *l*-epinephrine-d-bitartrate, and 1 ml of pH 8.3 borate buffer (0.5 M) were added to 10-ml portions of urine. The urine was adjusted with 3 N NaOH to pH 8.3 (measured with a glass electrode), and poured through the column. The column was washed with 50 ml of glass-distilled water containing 0.1 per cent EDTA, and the epinephrine was eluted with 20 ml of 1 N acetic acid. Aliquots of the acetic acid eluate were concentrated by lyophilization; 0.2 ml of distilled water and 10 ml of the ethanol-fluor were added, and the radioactivity was assayed as above. The recoveries of added epinephrine were 100 ± 5 per cent, measured by direct fluorimetry of the acetic acid eluates, and specificity of the eluted

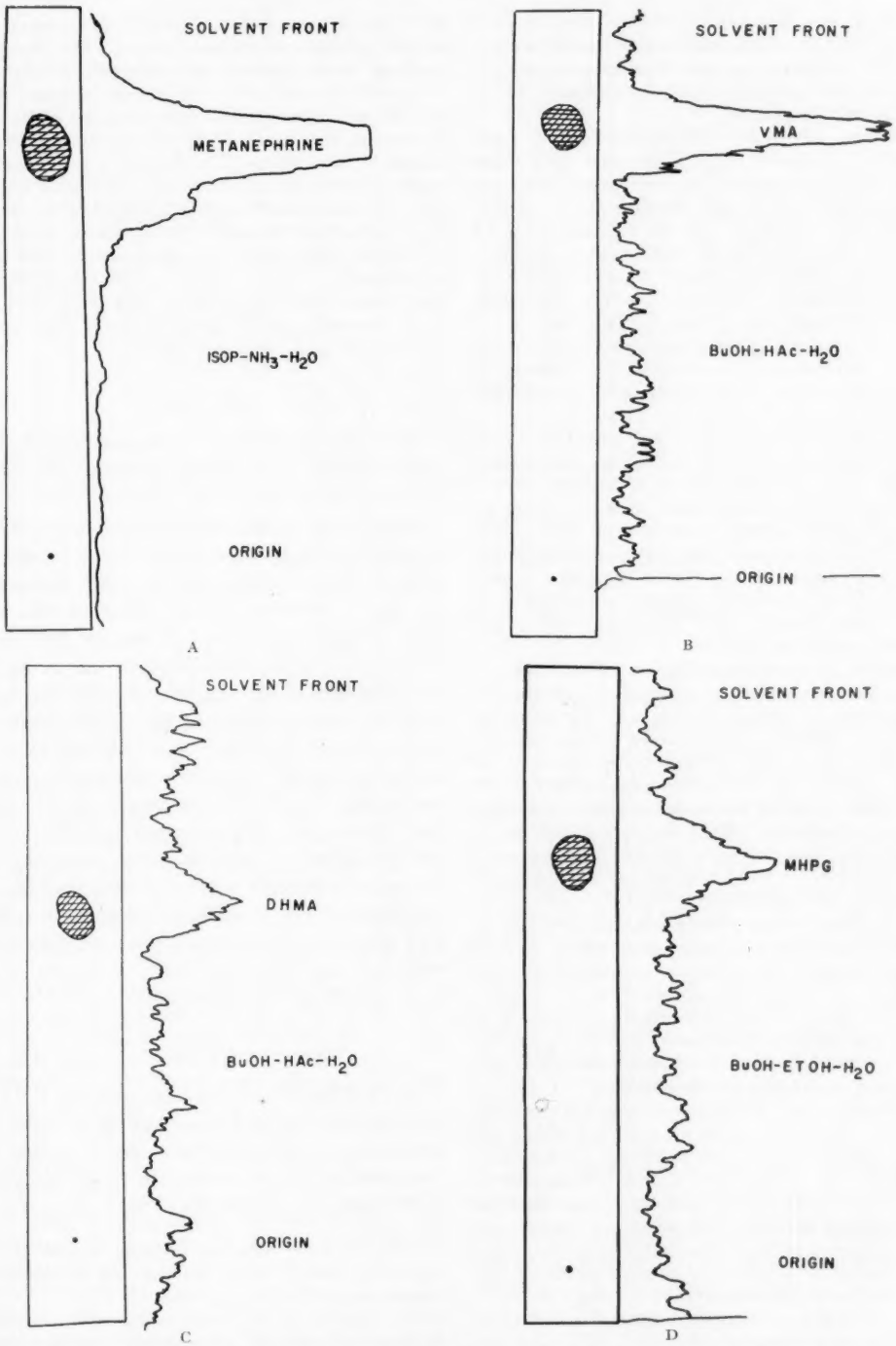

FIG. 1. RADIOGRAMS OF EXTRACTS OF URINE, COLLECTED AFTER 7-H^3-EPINEPHRINE INFUSION, SHOWING SPECIFICITY OF THE EXTRACTED MATERIAL. A) Metanephrine = 3-O-methyl-epinephrine; B) VMA = 3-methoxy-4-hydroxymandelic acid; C) DHMA = 3,4-dihydroxymandelic acid; D) MHPG = 3-methoxy-4-hydroxyphenylglycol.

radioactivity was demonstrated by the presence of a single peak of radioactivity corresponding to the locations of authentic epinephrine on paper chromatograms in *n*-butanol : acetic acid : water (4 : 1 : 1) and in phenol : 0.01 N HCl–SO_2 (80 : 20) systems.

Estimation of free 3,4-dihydroxymandelic acid (DHMA). One-tenth vol of 1 per cent EDTA and 0.02 vol of freshly prepared 2 per cent ascorbic acid solution were added to an aliquot containing about 1 μc total radioactivity in the urine. The pH was adjusted to 8.4 by the addition of 1 N NaOH (using a glass electrode), and the final volume was adjusted to 120 ml by the addition of glass-distilled water. The solution was centrifuged and 100 ml of the supernatant solution was poured through a 3 cm high, 0.4 cm diameter column of alumina (Woelm, nonalkaline). Ten ml of 0.2 M sodium acetate solution (pH 8.4) and 10 ml of glass-distilled water were also passed through the column in that order. The free catechols (epinephrine and DHMA) were eluted from the column with 30 ml of 0.2 N H_2SO_4. The sulfuric acid eluate was extracted with 5 vol of ethyl acetate. No epinephrine is extracted under these conditions, but 45 per cent of the DHMA passes into the ethyl acetate phase. The ethyl acetate extract was evaporated to dryness *in vacuo*, and the residue dissolved in 5 ml of ethanol. Four ml of the resulting ethanol solution was added to 10 ml of the toluene-fluor and the radioactivity was determined as mentioned above.

Estimation of conjugated DHMA. The conjugated DHMA was determined in the combined effluent and washings from the alumina column used for determination of free DHMA. The combined solution was adjusted to pH 1 with HCl and heated on a steam bath for 1 hour. After cooling, 0.1 vol of 1 per cent EDTA, 0.02 vol of freshly prepared 2 per cent ascorbic acid solution and 10 μg of authentic DHMA were added, and the resulting solution was adjusted to pH 8.4, chromatographed on alumina, extracted, concentrated and counted as described above for free DHMA.

The specificity of the procedure for DHMA was indicated by the finding of a major peak (85 per cent of radioactivity) at Rf 0.58 which corresponded to authentic DHMA on paper chromatograms developed in *n*-butanol : acetic acid : water (4 : 1 : 1) (see Figure 1C). A second smaller peak at Rf 0.81, increased after storage of the extract in the refrigerator for 1 day, indicating that it was a breakdown product of the DHMA.

Estimation of 3-methoxy-4-hydroxyphenylglycol (MHPG). One-tenth vol of the combined effluent and washing from the alumina column used in the determination of the free DHMA (see above) was adjusted to pH 11.5 with 3 N NaOH; solid $BaCl_2$ was added and the precipitated phosphate and sulfate were centrifuged until well packed at the bottom of the tube. An aliquot of the supernatant solution was adjusted to pH 6.5 with 3 N acetic acid, sulfatase (Glusulase, Endo Products, N. Y.) was added to a final concentration of 1,000 U per ml, and the mixture was incubated at 37° C for 24 hours. The solutions were centrifuged and an aliquot of the supernatant solution was poured onto a 7.5 cm column of Dowex-50 ion exchange resin.[2] The column was washed with an equal volume of water. The effluent and washings were combined, saturated with sodium chloride, and extracted with 5 vol of ethyl acetate. About 85 per cent of the MHPG was extracted under these conditions. An aliquot of the ethyl acetate extract containing at least 60 mμc of radioactivity was evaporated under a current of warm air in a scintillation counting vial,[3] the residue was dissolved in 14 ml of the ethanol-fluor mixture and counted. The specificity of this procedure was indicated by the presence of a single peak of radioactivity corresponding to authentic MHPG in the *n*-butanol : acetic acid : water (4 : 1 : 1), isopropanol : 7 N ammonia (4 : 1), and *n*-butanol : ethanol : water (4 : 1 : 1) systems (see Figure 1D).

RESULTS

The infused tritium was almost quantitatively excreted in the urine within 54 hours; the median value of excretion was 94.5 per cent. Water was distilled from several specimens of the urine collected after the H^3-epinephrine infusions and was assayed for tritium content. No measurable amount of radioactivity was found in the water of these urine specimens. It has also been established that the methods described above for the quantification of the individual metabolites do not result in measurable loss of the tritium label.

An average of 97 per cent (SE ± 4.0) of the tritium in the urine has been accounted for by the metabolites which were measured in this study (see Table I). The catechols, constituting 8.4 per cent of the tritium in the urine, are made up of epinephrine (6.8 per cent) and total DHMA (1.6 per cent). The 3-methoxy compounds totaled 89.2 per cent: these were free and conjugated metanephrine, 40.8 per cent; VMA, 41.2 per cent; and MHPG, 7.1 per cent.

DISCUSSION

In 11 of the 12 subjects listed in Table I, *d,l*-H^3-epinephrine was used; therefore, the urinary tritium came equally from the *d*- and *l*-isomers. At considerable cost in material a small quantity of *l*-H^3-epinephrine-d-bitartrate was obtained by

[2] The Dowex-50 resin was prepared by soaking overnight in 3 N NH_4OH and washing until the effluent was neutral when tested with pH paper.

[3] Low potassium, No-sol-vit glass liquid scintillation spectrometer vials, 20 ml capacity, with polyethylene snap-cap, were obtained from the Wheaton Glass Company, Millville, N. J.

TABLE I

Urinary metabolites following infusion of H^3-epinephrine in normal young men *

Subjects		Total dose *l*-Epi.		Total dose *d*-Epi.		Per cent of inf. tritium excreted	Metabolites expressed as per cent of tritium in the urine: ME Free	ME Gluc.	ME Sulfate	ME Total ME	VMA	Epi	MHPG	DHMA Free	DHMA Conj.	DHMA Total	Total of metabolites
no.		μg	μc	μg	μc												
2	R. A.	1,101	178	367	178	78	6	4	22	32	38	9	8.4	0.61	0.96	1.57	89
4	D. G.	1,012	173	337	173	84	6	4	24	34	41	7	5.4	0.76	1.10	1.86	89
6	R. C.	930	200	310	200	93	4	6	19	29	28	7	4.5	0.00	0.04	0.04	68
8	P. G.	1,064	182	355	182	100	6	6	34	46	36	7	6.1	0.77	1.00	1.77	97
10	R. B.	453	211	453	211	99	6	5	42	53	51	4	7.2	0.52	0.49	1.01	116
12	M. Y.	393	216	393	216	88	4	8	46	58	36	6	7.9	0.31	1.26	1.57	109
14	D. H.	445	207	445	207	96	7	5	27	39	59	5	5.9	0.81	1.31	2.12	111
16	M. D. N.	403	188	403	188	89	4	7	20	31	39	7	7.5	0.52	1.20	1.72	86
18	D. S.	311	158	23	158	100	4	8	21	33	48	7	7.6	1.14	0.65	1.79	97
20	L. S.	399	228	0	0	96	5	6	30	41	40	9	7.9	0.94	0.80	1.74	100
22	D. S. P.	410	199	410	199	103	6	5	31	42	34	5	9.1	0.66	1.22	1.88	92
24	D. W.	321	156	321	156	86	5	8	38	51	45	8	8.2	0.94	1.16	2.10	114
	Mean					92.6	5.2	6.0	29.5	40.8	41.2	6.8	7.1	0.66	0.93	1.60	97
	SD					7.7	1.1	1.5	9.0	9.5	8.4	1.5	1.4	0.31	0.38	0.57	14

* *l*-Epi and *d*-Epi =*l*- and *d*-epinephrine (free base); Inf. =infused; Gluc. =glucuronide; ME =metanephrine; VMA =3-methoxy-4-hydroxymandelic acid; MHPG =3-methoxy-4-hydroxyphenylglycol; DHMA =3,4-dihydroxymandelic acid; Conj. =conjugated.

resolution of the racemic material.[4] The *l*-H^3-epinephrine was used for the infusion of Subject 20 shown in Table I, in a schizophrenic patient infused in part of the study (12, 14), and in two subjects used by Kopin (16). In all four of these subjects in which the *l*-H^3-epinephrine was used, the distribution of the tritium among the epinephrine metabolites did not differ significantly from that found after the racemic H^3-epinephrine. This finding in man is in agreement with the finding of Schayer, Smiley and Kaplan in rats that the urinary chromatographic pattern of excretion was not significantly different after the *d*-and *l*-β-C^{14}-epinephrine (17).

Since Axelrod's demonstration of the O-methylation pathway of epinephrine metabolism in rats (5), the question of the presence and quantitative importance of this pathway in man has been of increasing interest. Resnick and Elmadjian (9) have reported that 34 per cent of the radioactivity of infused N-methyl-C^{14}-epinephrine was excreted in the urine of human subjects. This result indicated that approximately one-third of infused epinephrine did not lose its side chain. Since only 1 to 5 per cent of the infused epinephrine was excreted unchanged, the remaining 29 to 33 per cent must have been a metabolite with the side chain intact. Later studies by Resnick, Wolfe, Freeman and Elmadjian (18), Kirshner, Goodall and Rosen (10), and LaBrosse and co-workers (7), have shown that this metabolite is metanephrine, the O-methylation product of epinephrine. Kirshner and associates (19) have stated "that monoamine oxidase plays an important role in the inactivation of epinephrine at least in the short period of time after an infusion when it has physiological activity." This conclusion is difficult to accept because of the general lack of potentiation of the physiological effects of epinephrine by monoamine oxidase inhibitors. Furthermore, Kirshner and co-workers (19) base this conclusion on the finding that the radioactivity in the epinephrine fraction of the urine is doubled as a result of iproniazid treatment. However, calculation from their data reveals that this increase represents a change from 2.0 to 3.6 per cent of the infused epinephrine, and this change was found only during the initial

[4] The resolution was accomplished by adding 5.1 mg of nonradioactive *l*-epinephrine-d-bitartrate to 4.6 mg of *d,l*-H^3-epinephrine-d-bitartrate and dissoliving in 15 μl of water plus 90 μl of absolute methanol. After standing in the freezer at −10° C for three days, the vial was brought to room temperature and the slightly brownish solution was treated with charcoal. The tube was centrifuged, the supernatant solution was decanted into a 5 × 50 mm test tube, and the solvent was removed by distillation *in vacuo*. As the solution became concentrated, crystals formed in the slightly brownish, syrupy liquid on the sides of the tube. The crystals were washed with methanol and dried in a vacuum desiccator. The dry crystals weighed 0.88 mg (12 per cent yield). The small quantity of this material made optical rotatory measurements impractical; however, bioassay by rat blood pressure measurements indicated that it contained > 90 per cent *l*-epinephrine.

collection period. This small and evanescent difference in the excreted epinephrine, in view of the numerous variables involved, does not seem to merit the broad conclusion drawn. In contrast, the following evidence in the present report confirms previous reports from this laboratory (5–7) indicating that O-methylation is the principal pathway in the metabolism of circulating epinephrine. Following an infusion of β-H^3-epinephrine to a total of 12 human subjects, 40.8 per cent of total 54-hour urinary radioactivity has been found to be free and conjugated metanephrine. An indication of the proportion of metanephrine which is further metabolized to VMA and MHPG can be obtained by studying the excretion of these metabolites following infusion of labeled metanephrine. When 7-H^3-metanephrine was infused intravenously, 50 per cent of the radioactivity in the urine was found to be free and conjugated metanephrine and 25 per cent was found to be VMA. If it is assumed that the ratio of VMA to metanephrine, excreted after metanephrine is infused, is indicative of the manner in which endogenously formed metanephrine is metabolized, then it can be calculated that half of the VMA (or 20.6 per cent of the total urinary radioactivity) would have been formed via metanephrine after the 7-H^3-epinephrine infusion. In the present study an additional 3-methoxylated metabolite, MHPG, has been quantified. Kopin (16) has shown that about half of the latter metabolite, MHPG, as well as VMA, is formed by 3-O-methylation prior to deamination. Therefore, of the 7.1 per cent of the total urinary tritium which is H^3-MHPG in the study reported here, 3.6 per cent probably had undergone O-methylation to metanephrine prior to deamination to MHPG. Therefore, of the total urinary radioactivity, 40.8 per cent (free and conjugated metanephrine), 20.6 per cent (VMA formed from metanephrine) and 3.6 per cent MHPG formed from metanephrine) or a total of 65 per cent of the urinary radioactivity was in metabolites formed from the tritium-labeled epinephrine by 3-O-methylation as the first step in its metabolism. This finding agrees well with the 67 per cent O-methylation obtained in a definitive study by Kopin (16) in which *l*-7-H^3-epinephrine and *l*-3-methoxy-C^{14}-metanephrine were administered simultaneously to human subjects.

These findings present support for the hypothesis that 3-O-methylation is the principal pathway of metabolism for circulating epinephrine in man, at several pharmacological dosage levels as well as in the tracer dose (12.6 μg of *l*-epinephrine base), which was used by Kopin in the double-labeled experiments (16). It is of interest, that almost identical results were obtained with *l*- and *dl*-7-H^3-epinephrine in this and the experiments which utilized the double label (16).

Considering the question of whether monoamine oxidase or catechol-O-methyl transferase is the more important enzyme in the metabolic inactivation of epinephrine, Griesemer and colleagues (20) have reported that monoamine oxidase inhibitors do not potentiate the action of epinephrine. Also, Friend, Zileli, Hamlin and Reutter (21) have shown that administration of iproniazid (a monoamine oxidase inhibitor) does not alter the blood pressure, pulse rate, or other physiological effects of a norepinephrine infusion in man. It has been shown after labeled epinephrine that iproniazid decreases the urinary excretion of VMA, but increases the excretion of metanephrine (18). Hence, iproniazid does not inhibit catechol-O-methyl transferase. On the other hand, Bacq demonstrated many years ago that pyrogallol and several catechols potentiate the action of epinephrine (22). Recently, Bacq, Gosselin, Dresse and Renson have shown that catechol inhibits the action of O-methyl transferase on epinephrine *in vitro,* and they suggest that this inhibition is the mechanism for the sensitization of smooth muscle to epinephrine by catechols (23). Axelrod and Laroche have shown that pyrogallol is an effective inhibitor of catechol-O-methyl transferase, both *in vitro* and *in vivo* (24). From these reports it is known that pyrogallol and several catechols potentiate the action of epinephrine and simultaneously inhibit the action of the enzyme involved in the principal pathway of metabolism of this catechol amine. Furthermore, the O-methylated products are virtually inactive compared to the parent catechol amine (7, 25). These findings are compatible with the thesis that O-methylation is involved in the physiological inactivation of epinephrine and norepinephrine.

Within the past three years the recovery of infused, and presumably endogenous, epinephrine

in terms of known metabolites has increased from a few per cent to practically complete accountability, as shown in the present study. An opportunity is now provided for a more precise evaluation of the endogenous production of the catechol amine hormones or their abnormal degradation in various clinical states.

SUMMARY

The metabolism of 7-H^3-epinephrine-d-bitartrate was studied in normal young men. An average of 92.6 per cent (SD ± 7.7) of the infused radioactivity was excreted in the urine within 54 hours. The tritium-labeled metabolites were isolated, and gave the following percentages (mean ± SD) of total radioactivity in the urine: unchanged epinephrine, 6.8 ± 1.5; free metanephrine, 5.2 ± 1.1; metanephrine glucuronide, 6.0 ± 1.5; metanephrine sulfate, 29.5 ± 9.0; 3-methoxy-4-hydroxyphenylglycol, 7.1 ± 1.4; free 3,4-dihydroxymandelic acid (DHMA), 0.7 ± 0.3; and conjugated DHMA, 0.9 ± 0.4. The isolated metabolites averaged 97 per cent (SE ± 4.0) of the total urinary radioactivity.

The relevance of these findings to the routes of epinephrine metabolism is discussed and, in the light of present knowledge, they support the thesis that O-methylation followed by deamination is the major route of metabolic inactivation of circulating epinephrine.

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STUDIES OF ANTERIOR PITUITARY TISSUE *IN VITRO*: EFFECTS OF INSULIN AND EXPERIMENTAL DIABETES MELLITUS UPON CARBOHYDRATE METABOLISM *

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Since the classical experiments of Houssay and Biasotti (1), Evans, Meyer, Simpson and Reichert (2), Young (3), and Long and Lukens (4), the diabetogenic potential of pituitary hormones has been amply documented (5). However, the metabolism of glucose by the pituitary has received but little examination (6–9), and the authors have been unable to find any published studies of the hormonal determinants of hypophyseal carbohydrate economy. Because of the potential contribution of these factors to the endocrine function of the pituitary, experiments were initiated to define the role of insulin. The studies have demonstrated that the assimilation and disposition of glucose by surviving preparations of anterior pituitary from normal rats and beef may be enhanced by insulin *in vitro*. Moreover, it has been shown that glucose metabolism is significantly reduced in anterior pituitaries excised from rats rendered insulin-deficient by total pancreatectomy or alloxanization, and that these hypophyseal derangements can be prevented by replacement therapy with insulin *in vivo*.

METHODS

I. Preparation of tissues for incubation

A. Calf pituitary. Pituitary glands were obtained from male and female calves at the abattoir within 5 to 15 minutes after the death of the animals by exsanguination. The whole glands, left in their fascial investments, were placed in plastic bags and transported to the laboratory in an insulated container at a temperature of 4 to 10° C. Upon arrival at the laboratory, the capsules and adherent diaphragma sellae were removed with scissors and the glands were separated into anterior (AP) and posterior (PP) lobes by blunt dissection in Krebs-Ringer bicarbonate (KRB) at room temperature. A definite cleavage plane between the two portions (AP and PP) facilitated separation. This was documented histologically, and in calf the pars intermedia was included with the AP.

Slices of 0.7 to 1.0 mm thickness were prepared free-hand from calf AP which was held loosely between plastic blocks and supported on filter paper moistened with KRB. Although it was appreciated that gaseous diffusion into the center of the slice would probably be limited, thick slices were utilized for this study on the premise that insulin responsiveness *in vitro* might be enhanced by minimizing the cut surface area per unit weight of tissue. While this work was in progress, a report appeared which supported this assumption in studies of the effect of insulin upon rat spinal cord *in vitro* (10). Care was taken to pair the slices in control and experimental vessels with respect to gland topography since the various adenohypophyseal cell types are not uniformly distributed in the calf pituitary (11, 12). Calf AP was divided into hemilobes which were cut into four slices. The two slices representing the outer layers of the hemilobes were smaller in diameter, whereas two larger slices were derived from the central portion. To fill four incubation vessels from a single AP, one outer and one inner slice from opposite hemilobes were introduced into each vessel. The total calf AP averaged 393 ± 27 mg [1] and tissue weights in AP vessels ranged from 76 to 140 mg.

B. Rat pituitary. Male albino rats weighing 200 to 350 g were obtained from the Charles River Breeding Laboratories (Sprague-Dawley descendants) and maintained in this laboratory on Purina laboratory pellets. In all experiments, pituitaries were excised within 1 minute following sacrifice of animals by stunning and decapitation. Individual glands were placed directly in KRB at room temperature and AP and PP were teased apart with the aid of a dissection microscope (20×). Histological examination demonstrated complete separa-

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† Research Fellow of the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service.

[1] Mean ± standard error (SE).

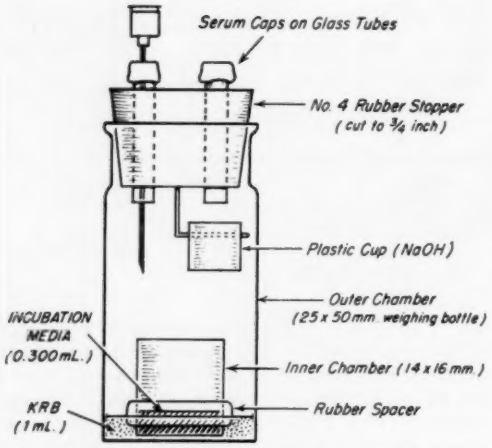

FIG. 1. MODIFIED REACTION VESSEL FOR INCUBATION OF SINGLE RAT ANTERIOR PITUITARY. For description, see text.

tion of AP and PP. The pars intermedia was present as adherent fragments about the posterior lobe.

For studies of pituitaries from normal fed donor rats, tissues from multiple animals were pooled. Each AP was divided at the midline with a sharp blade and each hemilobe was *a*) gently rolled on gauze, *b*) weighed on a 10 mg torsion balance accurate to ± 0.01 mg, and *c*) placed in either a "control" or "experimental" vessel (see below) for incubation. To fill two such paired vessels, a total of 6 to 10 rats was sacrificed serially. Thus, individual vessels contained the equivalent of 3 to 5 anterior pituitaries (16.4 to 20.0 mg). In paired vessels, the tissue weights were within ± 1 mg. On the other hand, for studies of hypophyseal carbohydrate metabolism in rats which had been subjected to experimental diabetes or other manipulations prior to sacrifice (see below), single anterior pituitaries were incubated individually. Herein, the intact AP was employed and hemisection was not instituted.

II. Incubation technique and media composition

In all of the studies, tissues were incubated for 3 hours at 38° C in a Dubnoff metabolic shaker (shaking rate 96 cycles per minute). The suspending medium consisted of KRB, pH 7.4, supplemented with unlabeled (National Bureau of Standards) and C^{14}-labeled glucose to final concentrations of 1.0 to 2.0 mg glucose per ml, and 0.8 to 3.0 μc C^{14} per ml. The gas phase consisted of 95 per cent O_2, 5 per cent CO_2. The various preparations of radioactive glucose, [i.e., labeled uniformly (C-U); or in the first (C-1) or sixth (C-6) carbon atoms] were obtained chromatographically pure from several commercial sources.[2] For studies of insulin action *in vitro* upon calf slices and pooled hemisected AP from normal rats, media were further supplemented with either 1.0 U per ml (40 μg) of "glucagon-free" crystalline beef insulin[3] ("experimental vessels") or 40 μg per ml of crystalline bovine albumin[4] ("control vessels"). In a few instances, albumin was omitted from the "control" systems (see below).

Incubations were conducted in 25×50 mm glass weighing bottles sealed with a rubber stopper (13). Two rubber-capped glass vents in the stopper permitted introduction of solutions or gases into the sealed vessels through needles. The stoppers also supported small plastic cups into which NaOH was introduced at the end of incubation for collection of $C^{14}O_2$ as outlined below. In the experiments with calf pituitary slices and pooled hemisected rat AP, the total volume of incubation medium consisted of 1.2 ml which was directly introduced into the weighing bottle. For incubation of individual rat anterior pituitaries, the flasks were modified as shown in Figure 1. A small glass cup measuring 14×16 mm (inner chamber, Figure 1) was placed in the larger bottle. A rubber ring (cut from rubber tubing) was slipped around the small vessel to prevent capillary migration of fluid between the inner and outer chambers. Intact single adenohypophyses were incubated in the inner chamber and suspended in a total volume of 0.3 ml KRB. To minimize evaporative losses in this system and to promote conduction of heat from the Dubnoff bath to the inner chamber, an additional 1.0 ml of isosmotic KRB was placed into the outer chamber.

Incubation was terminated by introducing 0.2 ml 5 N NaOH into the plastic cups, and thereafter either *a*) introducing 0.1 ml 1 N HCl directly into the 1.2 ml of suspending medium, or *b*) introducing 1.0 ml of 0.03 N HCl in isotonic NaCl into the inner chamber and 0.1 ml 1 N HCl into the outer chamber. Two hours was allowed for evolution of $C^{14}O_2$ after acidification of the media.

III. General analytical techniques

A. Glucose. Protein-free filtrates were prepared from plasma or whole blood by precipitation with $Ba(OH)_2 + ZnSO_4$ (14). Plasma was obtained from heparinized blood collected from the severed neck vessels at sacrifice. In some experiments, whole blood was obtained from the tail vein prior to sacrifice. The filtrates were analyzed for glucose by the enzymatic colorimetric method of Huggett and Nixon (15). Aliquots of suspending media which had been incubated both with and without tissues were similarly precipitated with $Ba(OH)_2 + ZnSO_4$, and the protein-free filtrates were employed for duplicate assay of glucose concentration.

B. Radioactive carbon dioxide. $C^{14}O_2$ evolved during the incubation period was precipitated quantitatively with

[2] Glucose-U-C^{14} purchased from Tracerlab and New England Nuclear Corporation, Boston, Mass. Glucose-1-C^{14} and glucose-6-C^{14} purchased from Nuclear-Chicago, Ill.

[3] Crystalline zinc insulin (lot no. 499667) assaying 25 U per mg was generously provided by Dr. O. K. Behrens and Dr. C. W. Pettinga of Eli Lilly Company.

[4] Armour lot no. T 68204.

barium chloride from sodium hydroxide and assayed in duplicate as barium carbonate, as described previously (16).

C. Glycogen C^{14}. At the end of incubation, calf AP slices were placed directly in 1 ml of 30 per cent KOH and 1 ml of 95 per cent enthanol and autoclaved for one hour (at 15 lbs 120° C). Carrier glycogen, 12.0 mg, was added and glycogen was precipitated by the method of Good, Kramer and Somogyi (17). The glycogen was precipitated a second time and then dissolved in 2.5 ml of distilled water for 24 hours of dialysis at 4° C against three changes of 100 vol of 0.001 M phosphate buffer (pH 7.4). The dialyzed glycogen solutions were then decanted and duplicate 1.0 ml aliquots were deposited on weighed cup planchets for radioactive assay in a gas-flow counter. Mass absorption corrections were applied.

The dialysis method was validated for calf anterior pituitary by comparing the specific activity of glycogen obtained by this method with the specific activity of thrice recrystallized glucosazones, prepared from an acid hydrolysate of the parent glycogen solution. In four experiments, the glucosazone and dialysis methods yielded similar results. Control studies also indicated that glycogen radioactivity was not measurably contaminated with cerebrosides (18).

D. Lipid C^{14}. In experiments with pooled AP tissue from normal rats, total chloroform : methanol-extractable lipids ($CHCl_3$: MeOH lipid C^{14}) (19) were obtained for radioactive assay as follows. After incubation, the tissues were placed directly into chloroform : methanol 2 : 1 (vol/vol) and homogenized. The mixture was centrifuged, the supernatant fluid decanted, and the sediment re-extracted in warm (60°C) chloroform : methanol. The combined extracts were washed three times with isotonic NaCl (19) and assayed at infinite thinness on cup planchets in a gas-flow counter. For calf AP and a small number of experiments with rat AP, tissue lipids were fractionated into nonsaponifiable lipids, and long chain fatty acids (20). The nonsaponifiable lipid extract was reduced in volume and quantitatively plated for radioactive assay in a micromil-window gas-flow counter. The fatty acids were evaporated under reduced pressure and redissolved in toluene, 0.4 per cent 2,5-diphenyloxazole (PPO) and 0.005 per cent 1,4-*bis*-2-(5-phenyloxazolyl)-benzene (POPOP)[5] solution for radioactive assay in a Packard Tri-Carb liquid scintillation counter.[6]

IV. Expression of results

Net glucose assimilation in micrograms of glucose per milligram of tissue was calculated from the initial tissue wet weight and the total micrograms of glucose disappearing from the media during 3 hours of incubation. The latter value was calculated on the basis of the difference between the final content of glucose in experimental vessels and in vessels which had been incubated without tissue.

Disposition of glucose-C^{14} was expressed as micrograms of glucose-C^{14} per milligram of initial wet tissue weight and calculated on the basis of the specific activity of the glucose in the initial suspending medium and the recovered C^{14}. Initial specific activity was determined for each experiment by separate chemical and radioactive assay of the media in duplicate blank vessels. Sufficient counts were collected to reduce random counting error to less than ± 1 per cent for standards, $C^{14}O_2$, and glycogen-C^{14}, and to less than ± 3 per cent for the lipid fractions. Since the authors were unable to demonstrate glucose-6-phosphatase activity in calf anterior pituitary tissue (21), the values for glucose assimilation and initial specific activity of the medium could be employed to assess net assimilation of glucose radioactivity.

The statistical significance of the effects of insulin *in vitro* was evaluated by testing the ratios of the observed values in paired "control" and "experimental" vessels of individual experiments for deviation from a ratio of unity. The common logarithms of the ratios were employed and Student's *t* values were calculated from the mean and standard error of the mean of the log form of these ratios (22). Such analysis of relative changes effected by insulin made it possible to pool data from multiple experiments conducted with varying concentrations of glucose in the medium.

TABLE I

The effect of insulin in vitro upon glucose assimilation by slices of calf anterior pituitary

Reaction mixture: 76 to 140 mg tissue slices suspended in 1.2 ml KRB containing 2.0 mg glucose per ml and either 1.0 U, 40 μg, insulin per ml (+) or 40 μg crystalline bovine albumin per ml (−). Incubation: 3 hours, 38° C.

Gland	Glucose assimilation (−)	Glucose assimilation (+)	Insulin effect
	μg/mg		+/− ×100*
1	3.28	3.65	111.3
2	4.07	4.79	117.7
3	5.69	7.25	127.4
4	4.73	7.94	167.9
5	8.49	7.58	89.3
6	6.20	7.81	126.0
7	5.50	7.01	127.5
8	5.44	6.35	116.7
9	1.34	2.08	155.2
10	5.74	5.93	103.3
11	1.53	1.49	97.4
12	3.27	5.00	152.9
13	3.76	5.31	141.2
14	4.76	4.88	102.5
15	5.00	6.48	129.6
16	2.97	4.00	134.7
17	3.79	4.30	113.5
		Mean ± SE	124.4 ± 4.5
		p	0.001

* Insulin effect: results observed in insulin-containing systems (+) expressed as a percentage of the values in control (−) vessels. By this convention, 100 denotes no effect.

[5] Purchased from Pilot Chemical Co., Watertown, Mass.

[6] Packard Instrument Company, Inc., La Grange, Ill.

TABLE II

Metabolism of glucose-U-C^{14} by calf anterior pituitary

Reaction mixture: as in Table I; glucose 2.0 mg per ml labeled with 0.8 to 1.2 μc glucose-U-C^{14} per ml.
Incubation: as in Table I.

Gland no.*	Glucose-U-C^{14} (μg/mg) to: CO_2 (−)	CO_2 (+)	Glycogen (−)	Glycogen (+)	Non-sap. lipid (−)	Non-sap. lipid (+)	Fatty acid (−)	Fatty acid (+)
6	1.12	1.35	0.72	1.01	0.0028	0.0040	0.025	0.41
7	0.76	1.17	0.64	0.91	0.0024	0.0045	0.036	0.042
8	0.81	1.17	0.77	0.83	0.0027	0.0040	0.044	0.058
9	0.24	0.33	0.14	0.21	0.0006	0.0007	0.0026	0.0031
10	0.85	0.85	0.76	0.82	0.0032	0.0029	0.021	0.031
11	0.41	0.36	0.20	0.21	0.0011	0.0008	0.0048	0.0061

* Gland numbers as in Table I. Values for total glucose assimilation from the suspending medium in the absence (−) and presence (+) of insulin are shown in Table I.

RESULTS

I. Effects of added insulin upon carbohydrate metabolism of surviving anterior pituitary tissue from normal animals

A. Calf. Seventeen separate experiments were performed in which slices of adenohypophyses from slaughterhouse calves were incubated for 3 hours in 1.2 ml of media containing 2 mg glucose per ml (Table I). Under these conditions, net assimilation of glucose from the suspending media averaged 4.44 ± 0.43 μg per mg tissue in "control" vessels. With 13 of the 17 glands, glucose uptake was increased at least 10 per cent above control values by the addition of 1.0 U of insulin per ml. For all experiments, the induced augmentation of glucose assimilation averaged 24.4 per cent and was highly significant ($p = 0.001$, Table I).

In 12 of the 17 glands, intracellular disposition of some of the assimilated glucose was evaluated with glucose-C^{14}. Uniformly labeled glucose was used in six instances (i.e., glands 6 through 11). Herein, $C^{14}O_2$ was employed as an index of oxidative degradation, and anabolic utilization was assessed on the basis of the incorporation of radioactivity into glycogen, nonsaponifiable lipids, and fatty acids. Results are summarized in Table II. In the four experiments in which insulin promoted glucose uptake (i.e., 6 through 9), oxidative decarboxylation, glycogenesis and lipogenesis were also augmented. Contrariwise, in glands 10 and 11, in which insulin did not significantly affect the assimilation of glucose, uniform effects upon the disposition of glucose-U-C^{14} were not observed.

In six experiments (i.e., glands 12 through 17), differentially labeled preparations of radioactive glucose were employed (Table III). The existence of the phosphogluconic acid pathway for glucose oxidation in anterior pituitary tissue was demonstrated by the routine evolution of greater quantities of $C^{14}O_2$ from glucose labeled in the first carbon than from glucose labeled in the sixth position. In control vessels, C-1/C-6 ratios for $C^{14}O_2$ averaged 2.04 ± 0.17. Addition of insulin caused an average 14.8 per cent increase in $C^{14}O_2$ from glucose-1-C^{14} ($p = 0.1$) and an average 50.9 per cent increase in $C^{14}O_2$ from glucose-6-C^{14} ($p = 0.01$, Table III).

B. Rats. Eighteen experiments were performed with pooled tissues from normal fed rats. Paired groups of hemisected anterior pituitaries were incubated for 3 hours in 1.2 ml of media containing 1.0 to 2.0 mg of glucose per ml and 1.1 to 2.6 μc glucose-U-C^{14} (Table IV). Results from all experiments were combined in order to assess the statistical significance of changes effected by supplementing suspending media with 1.0 U of insulin per ml. In the presence of insulin, the mean assimilation of glucose was 16.7 per cent greater than control values ($p = 0.02$, Table IV). Concomitantly, the evolution of $C^{14}O_2$ from glucose-U-C^{14} was increased an average of 6.4 per cent ($p = 0.02$) and the mean incorporation of radioactivity into total chloroform-methanol extractable lipids was augmented 19.0 per cent ($p = 0.01$).

II. Carbohydrate metabolism of surviving anterior pituitary tissue from insulin-deficient rats

In this series of experiments, rats were subjected to various manipulations *in vivo* designed

TABLE III

Metabolism of glucose-1-C^{14} and glucose-6-C^{14} by calf anterior pituitary

Reaction mixture: as in Table I; glucose 2.0 mg per ml labeled with 1.1 and 1.3 μc per ml of glucose-1-C^{14} and glucose-6-C^{14}, respectively.
Incubation: as in Table I.

Gland no.*	Control (−) $C^{14}O_2$ (μg/mg) C-1	C-6	Ratio C-1/C-6	Insulin (+) $C^{14}O_2$ (μg/mg) C-1	C-6	Ratio C-1/C-6
12	2.04	1.34	1.52	2.74	2.39	1.14
13	2.64	1.55	1.70	3.31	2.10	1.58
14	1.62	0.686	2.37	1.77	1.12	1.58
15	1.90	0.811	2.34	2.02	1.57	1.29
16	1.01	0.552	1.83	1.49	0.794	1.88
17	1.68	0.679	2.48	1.34	0.737	1.82
	Mean ± SE		2.04 ± 0.17			1.55 ± 0.12

C-1 (−) vs C-1 (+) $p > 0.1$
C-6 (−) vs C-6 (+) $p < 0.01$
C-1/C-6 (−) vs C-1/C-6 (+) $p < 0.02$

* Gland numbers as in Table I.

TABLE IV

The effect of insulin in vitro upon the metabolism of glucose-U-C¹⁴ by rat anterior pituitary

Reaction mixture: 16 to 20 mg of paired, hemisected anterior pituitaries from normal fed rats suspended in 1.2 ml KRB containing 1.0 to 2.0 mg glucose per ml. Glucose labeled with 1.1 to 2.6 μc of glucose-U-C¹⁴. Media further supplemented with either 1.0 U, 40 μg, insulin per ml (+) or 40 μg crystalline bovine albumin per ml (−).
Incubation: 3 hours, 38° C.

Medium glucose concentration	Experiment	Glucose assimilation (−)	Glucose assimilation (+)	Insulin* effect (+/−×100)	$C^{14}O_2$ (−)	$C^{14}O_2$ (+)	Insulin* effect (+/−×100)	$CHCl_3$-MeOH lipid C^{14} (−)	$CHCl_3$-MeOH lipid C^{14} (+)	Insulin* effect (+/−×100)
mg/ml		*μg/mg*			*μg/mg*			*μg/mg*		
1.0	1†	7.2	9.0	125.0	1.60	1.46	91.3	0.043‡	0.056‡	130.2
	2†	6.8	10.7	157.4	1.30	1.43	110.0	0.039‡	0.055‡	141.0
	3†	9.1	10.9	119.8	2.14	2.57	120.1	0.165	0.193	117.0
	4†	10.4	10.5	101.0	2.12	2.12	100.0	0.146	0.180	123.3
	5	10.2	9.2	90 2	2.45	2.16	88.2	0.186	0.205	110.2
	6	10.3	10.4	101.0	3.14	3.10	98.7	0.220	0.252	114.5
1.1	7	11.8	14.7	124.6	3.09	3.01	97.4	0.224	0.300	133.9
	8	10.7	14.6	136.4	2.76	3.46	125.4	0.251	0.352	140.2
1.5	9	13.5	16.6	123.0	2.52	2.57	102.0	0.212	0.261	123.1
	10	13.1	13.9	106.1	1.98	2.23	112.6	0.220	0.180	81.8
2.0	11	20.4	16.6	81.4	4.14	4.87	117.6	0.200	0.273	136.5
	12	19.8	16.9	85.4	3.76	3.79	100.8	0.192	0.201	112.3
	13	16.7	25.7	153.9	3.78	3.84	101.6	0.219	0.244	111.4
	14	15.3	16.9	110.5	3.81	3.86	101.3	0.179	0.201	112.3
	15	21.1	21.5	101.9	2.91	3.40	116.8	0.200	0.257	128.5
	16	8.2	18.8	229.3	3.11	3.59	115.4	0.210	0.221	105.2
	17	21.2	24.3	114.6	3.65	3.81	104.4	0.181	0.233	128.7
	18	11.4	12.4	108.8	3.19	3.89	121.9	0.203	0.234	115.3
		Mean ± SE		116.7 ± 6.0			106.4 ± 2.4			119.0 ± 3.3
		p		0.02			0.02			0.01

* Insulin effect: results observed in insulin-containing systems (+) expressed as a percentage of the values in control vessels (−).
† Albumin omitted from control vessels.
‡ Fatty acids.

to alter levels of circulating insulin. Thereafter, anterior pituitaries from individual rats were excised and separately incubated in 0.3 ml KRB containing 2.0 mg glucose per ml, 3.0 μc glucose-U-C^{14} per ml and no supplemental albumin or insulin. Adenohypophyses were examined in the intact state, and were not hemisected as in the above studies with pooled tissues from normal animals.

A. Rats with total pancreatectomy. Total (99.5 per cent) pancreatectomy was performed as described by Scow (23) upon rats weighing between 220 and 330 g and fasted from 12 to 20 hours prior to operation. Comparable rats, matched by weight in individual experiments, were subjected to either: *a*) sham pancreatectomy, consisting of laparotomy and exposure of stomach, liver, duodenum and spleen to air for a similar period of time; or *b*) partial pancreatectomy, removing only the "first portion" (23) of the pancreas.

Additional control groups were prepared by removing a short section of bile duct to produce bile peritonitis or by removing 4.0 ml of blood from the aorta and injecting this blood into the peritoneal cavity to produce conditions of intra-abdominal hemorrhage with shock.

All rats were given 5.0 ml of saline and 12,000 U of crystalline penicillin subcutaneously after operation. The rats were maintained in individual metabolic cages until sacrifice, 20 hours following surgery. At that time, anterior pituitaries were excised and incubated individually.

To preclude the effects of fasting in the postoperative period, and hypoglycemia in animals which were treated with insulin (see below), glucose was administered to all of the rats subcutaneously. A total dose of 0.5 g glucose per 100 g body weight was administered as 10 per cent glucose in water in three divided doses at 6-hour intervals during the 20-hour postoperative period. The last injection was routinely administered 1 to 2 hours prior to sacrifice.

Some of the pancreatectomized animals were treated with insulin during the postoperative period. Therapy consisted of 0.5 U of protamine zinc insulin and 0.5 U of regular insulin subcu-

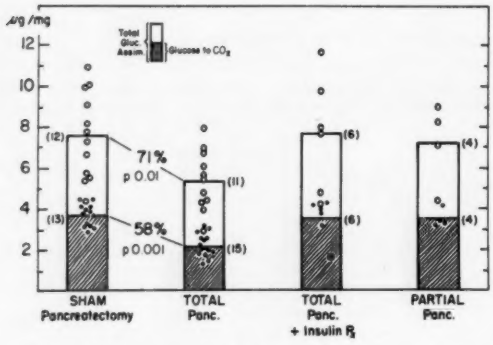

FIG. 2. TOTAL ASSIMILATION OF GLUCOSE AND MICROGRAMS PER MILLIGRAM OF GLUCOSE-U-C^{14} OXIDIZED TO $C^{14}O_2$ DURING INCUBATION OF SINGLE ANTERIOR PITUITARIES REMOVED FROM RATS 20 HOURS AFTER SURGICAL MANIPULATION. Mean glucose assimilation is indicated by the total heights of the bars; the shaded areas denote mean values for glucose oxidation to CO_2. Individual values for these parameters are indicated by the open and closed symbols, respectively. The single circled value in the totally pancreatectomized, insulin-treated group represents the result in the animal in whom normoglycemia was not restored by therapy (see text). Mean values for the percentile reductions in glucose utilization by the totally pancreatectomized rats and the statistical significance of these changes are indicated on the illustration. Numbers in parentheses indicate the number of animals in each category.

taneously at 5 hours and 0.1 U of regular insulin at 18 hours after operation.

Measurements of plasma glucose were employed to assess the severity of the experimental diabetes and the adequacy of the replacement therapy with insulin. Values were obtained 5 hours following surgery (i.e., prior to the administration of the first dose of glucose and before the exhibition of insulin in the insulin-treated group) and at sacrifice (i.e., following three doses of glucose and insulin therapy in some animals). Plasma glucose averaged 272 ± 33 mg per 100 ml [4][7] in untreated pancreatectomized rats 5 hours after operation and 762 ± 110 mg per 100 ml [15] at sacrifice following three doses of glucose. In five of six similar pancreatectomized rats (mean plasma glucose 270 ± 29 [6], 5 hours postoperatively), the exhibition of insulin in association with glucose reduced blood sugar to an average of 75 ± 11 mg per 100 ml [5] at sacrifice. Insulin therapy was inadequate in the sixth animal and final blood sugar was 187 mg per 100 ml. Significant abnormalities in blood sugar were not observed at any time intervals in the partial or sham pancreatectomized groups. Respective values for blood sugar in these groups were 84 mg per 100 ml [2] and 58 ± 4 mg per 100 ml [5] 5 hours following surgery, and 107 ± 5 mg per 100 ml [4] and 102 ± 6 mg per 100 ml [13] at sacrifice.

Patterns of carbohydrate metabolism observed during the *in vitro* incubations of individual anterior pituitaries are summarized in Figures 2 and 3. In untreated pancreatectomized rats, total assimilation of glucose, oxidation of glucose-U-C^{14} (Figure 2) and conversion of glucose-U-C^{14} to fatty acids (Figure 3) by AP, were significantly reduced as compared to values obtained with AP

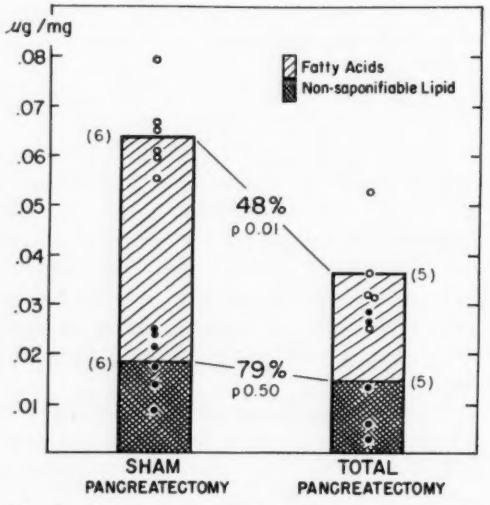

FIG. 3. INCORPORATION OF GLUCOSE-U-C^{14} INTO NONSAPONFIABLE LIPIDS AND FATTY ACIDS DURING INCUBATION OF SINGLE ANTERIOR PITUITARIES REMOVED FROM RATS 20 HOURS AFTER SURGICAL MANIPULATION. The total heights of the bars represent the sum of the micrograms per milligram of glucose-U-C^{14} recovered as fatty acids (diagonal lines) and nonsaponifiable lipids (cross-hatched). Mean values for the percentile reductions in lipogenesis in totally pancreatectomized rats and the statistical significance of these changes are indicated on the illustration. Numbers in parentheses denote the number of animals in each category.

[7] Throughout this paragraph, the blood sugar values given are expressed as the mean ± standard error in the respective groups. Brackets denote the number of animals in each experimental group from which blood was sampled. Five-hour specimens of blood were obtained from the tail vein. Bloods at sacrifice were collected from the neck following decapitation of the animals.

TABLE V

The effect of severe operative trauma upon the glucose metabolism of rat anterior pituitary

Reaction mixture: single intact AP obtained from rats 20 hours after operation and suspended in 0.3 ml KRB containing 2.0 mg glucose per ml labeled with 3.0 μc glucose-U-C^{14} per ml.
Incubation: 3 hours, 38° C.

Operative group	Rat		Glucose assimilation	$C^{14}O_2$
			μg/mg	μg/mg
A. Pancreatectomized rats				
Pancreatectomized + Complications		Mean ± SE	5.63 + − 1.23 [7]*	2.16 ± 0.14 [10]
Pancreatectomized + No complications		Mean ± SE	4.51 + − 1.65 [4]	2.32 ± 0.15 [5]
B. Control rats				
Bile peritonitis	1		9.78	4.48
	2		8.57	4.31
	3		11.25	3.99
	4		9.09	3.92
		Mean ± SE	9.67 ± 0.58	4.18 ± 0.13
Intra-abdominal hemorrhage	1		11.26	3.88
	2		8.75	3.98
	3		10.00	4.17
	4		11.46	4.14
		Mean ± SE	10.37 ± 0.63	4.04 ± 0.06
Sham	1		9.53	3.25
	2		8.45	4.11
	3		10.24	3.78
		Mean ± SE	9.41 ± 0.52	3.71 ± 0.25

* Number of animals in brackets.

from sham-operated animals. The values for these three parameters of glucose metabolism were reduced to 71 ($p = 0.01$), 58 ($p = 0.001$) and 48 ($p = 0.01$) per cent of the control values, respectively. Reduction of glucose metabolism was not observed with AP derived from the partially pancreatectomized rats or the five pancreatectomized rats in which replacement therapy with insulin had been adequate (Figure 2).

Many of the pancreatectomized animals displayed operative complications consisting of bile peritonitis or intra-abdominal hemorrhage. Although these phenomena did not appear to alter AP metabolism in pancreatectomized animals (Table V, A), separate experiments were performed to assess the effects of operative complications independent of disturbances in carbohydrate metabolism. Bile peritonitis or intra-abdominal hemorrhage with shock was produced in normal animals, and, after 20 hours, AP glucose metabolism was compared with that of sham-operated rats (Table V, B). Despite oliguria and prostration comparable in severity to that seen in pancreatectomized animals with operative complications, the AP metabolism of glucose was indistinguishable from that of the concurrently examined sham-operated control population. Thus, the observed depression in the metabolism of glucose by AP of pancreatectomized rats cannot be ascribed to operative trauma or shock.

The occurrence of operative complications in two-thirds of the pancreatectomized rats also afforded a means of assessing whether the derangement of pituitary metabolism was a primary consequence of hypophyseal insulin deprivation or whether it was secondary to generalized metabolic disturbances (i.e., acidosis, ketosis, triglyceridemia and so forth) resulting from insulin deprivation elsewhere. Gross stigmata of aberrant lipid metabolism (24) were not so pronounced in the animals with operative complications and surgical shock (25). Correlations were attempted by visually grading 0 to 4 + : *a*) fatty infiltration of the liver, *b*) turbidity of plasma, and *c*) semiquantitative chemical estimation of plasma "acetoacetic acid and acetone" (26). The metabolism of

glucose by excised AP did not correlate in severity with any of these indices of generalized metabolic abnormalities.

In the above studies of experimental diabetes, values for glucose assimilation by AP from sham-operated control animals (Figure 2 and Table V) were considerably lower than the glucose uptakes observed during incubation of pooled hemisected AP from normal fed rats in media similarly containing 2 mg per ml of glucose (Experiments 11–18, Table IV). The reasons for this are not immediately apparent, especially since comparable differences did not obtain in the evolution of $C^{14}O_2$ (Figure 2 and Table V vs Table IV). However, in any analysis, it should be noted that experimental conditions were not identical. Thus, the studies differed not only in the dietary and manipulative preparation of donor animals but also in the volume of suspending medium (i.e., 0.3 vs 1.2 ml), in the nature of suspending medium (i.e., plain KRB vs KRB + 40 μg albumin per ml), and in the status of the tissues (i.e., intact vs hemisected AP). That such subtle factors might influence assimilative function is suggested by the demonstration by Roberts and Keller (7) of appreciable anaerobic glycolysis in rat AP. In any event, the findings would suggest that normal values for glucose assimilation by surviving preparations of AP can only be defined within a rigid framework of given *in vitro* experimental conditions.

B. Alloxanized rats. Chronic diabetes mellitus was induced in rats by the subcutaneous administration of 125 mg alloxan monohydrate per kg body weight after a 24-hour fast. Six weeks after administration of alloxan, 12 diabetic rats were paired by weight and fasting blood glucose concentration (Table VI). Thereafter, all rats were allowed food *ad lib.* and one member of each pair was treated with insulin for the 7 days preceding sacrifice. Treatment consisted of single daily injections of 1.0 to 3.0 U of protamine zinc insulin per 100 g body weight adjusted to prevent urine glucose concentrations above 0.5 per cent. Two hours prior to sacrifice, the treated rats were given 4.0 U of regular insulin subcutaneously. The untreated rats gained little weight and exhibited uniformly high plasma glucose levels at sacrifice. The treated animals gained an average of 10 g body weight per day and had normal or moderately hypoglycemic levels of blood glucose at sacrifice (Table VI).

At the end of the treatment period, the anterior pituitary glands from all of the rats were incubated individually with glucose-U-C^{14}. The mean glucose assimilation and oxidation of glucose-U-C^{14}

TABLE VI

The metabolism of glucose by anterior pituitary in untreated and treated alloxan-diabetic rats

Reaction mixture: as in Table V.
Incubation: as in Table V.

Group	Pair	Body wt		Plasma glucose		Metabolism of glucose-U-C^{14} by AP	
		Initial	Sacrifice	Initial (fasting)	Sacrifice (fed)	Glucose assimilation	$C^{14}O_2$
		g	*g*	*mg%*	*mg%*	*μg/mg*	*μg/mg*
Alloxan: untreated	1	230	235	452	754	6.34	2.84
	2	325	375	358	498	8.20	2.71
	3	270	310	340	723	11.14	2.95
	4	330	325	312	558	6.71	3.11
	5	355	385	180	526	6.73	2.82
	6	370	385	161	481	6.41	3.08
	Mean ± SE	313	336	301	590	7.59 ± 0.76	2.92 ± 0.07
Alloxan: treated	1	300	375	423	72	11.95	4.06
	2	250	335	370	40	11.84	3.08
	3	360	400	336	49	9.62	3.83
	4	225	330	292	74	7.20	3.84
	5	335	400	231	36	9.49	3.60
	6	355	425	148	38	8.34	3.57
	Mean ± SE	304	378	300	52	9.74 ± 0.77	3.66 ± 0.14
				Untreated as % of treated		78	80
					p	0.1	0.001

to $C^{14}O_2$ by AP in untreated rats were 78 and 80 per cent, respectively, of the corresponding values in the treated group. The difference in $C^{14}O_2$ production was statistically significant ($p = 0.001$).

In this, as in all other studies, metabolic activity *in vitro* has been related to a unit of mass, i.e., milligrams of anterior pituitary. The validity of comparing glands of different weight in this fashion was examined by plotting oxidation of glucose to $C^{14}O_2$ as a function of total AP weight (Figure 4). This indicated that activity per unit mass within each experimental group remained relatively constant despite wide variations in pituitary mass. Thus, at any single level of AP weight, the total activity of AP from treated or sham-operated animals exceeded the values observed in untreated diabetic rats. Since AP : total body weight ratios in the animals with experimental diabetes mellitus did not significantly deviate from normal, it may be inferred that reduction of activity per unit mass represented a valid index of reduction in total pituitary activity.

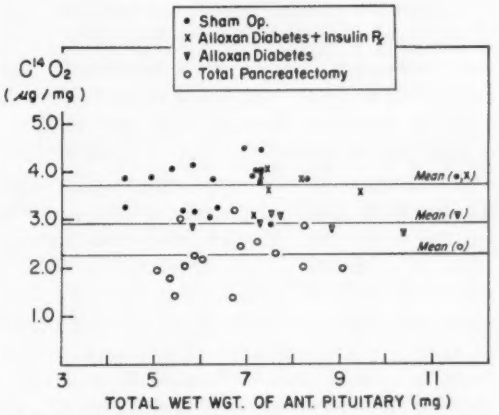

FIG. 4. THE RELATIONSHIP BETWEEN TOTAL ANTERIOR PITUITARY WEIGHT AND OXIDATION OF GLUCOSE-U-C^{14} TO $C^{14}O_2$ PER MILLIGRAM OF INCUBATED ANTERIOR PITUITARY TISSUE. Symbols denote individual values in the sham-operated, diabetic, and treated diabetic categories. Horizontal lines denote mean values for glucose oxidation in the various groups.

DISCUSSION

In establishing that a tissue is responsive to insulin and that the responsiveness is of physiological significance, multiple criteria should be fulfilled. First and ideally, one should be able to elicit a direct effect with insulin *in vitro*. This desideratum need not always obtain. For example, although liver slices do not usually respond to added insulin (*27*, *28*), mounting evidence would indicate that the hormone can directly modify hepatic function in the intact animal (*29*, *30*) or in the perfused liver (*31*). Furthermore, even when an action of insulin can be demonstrated *in vitro*, the relationship between dose and response need not equate with sensitivity to insulin *in vivo*. Many tissues contain soluble insulin-degrading enzymes which are leached into the suspending medium during incubation of cut preparations (*32*, *33*). Recent work in this laboratory has indicated that the resultant extracellular proteolysis of the added insulin diminishes its manifest effectiveness upon the incubated tissue (*34*). Moreover, besides such leaching artifacts, one might anticipate that the response of an insulin-sensitive tissue to exogenous insulin *in vitro* could be inversely conditioned by its exposure to *endogenous* insulin prior to excision from the donor animal. In the present studies, experimental design was influenced by these considerations. Thus, because "insulinase" (*35*) and other proteases (*36*) are present in anterior pituitary, and because hypophyseal vascularity may be sufficient to effect near maximal delivery of insulin to AP in the intact animal, "supramaximal" doses of 1 U per ml were employed for investigation of insulin addition *in vitro*. Precise dose-response relationships were not examined. Rather, attempts were made to assess only whether anterior pituitary is or is not capable of directly responding to insulin. Within this framework, unequivocal effects were obtained with slices of calf AP, and smaller, although still statistically significant, changes were observed with pooled hemisected AP from normal fed rats. Insofar as added insulin definitely affected the carbohydrate metabolism of surviving pituitary tissue, the *in vitro* studies demonstrated the existence of adenohypophyseal components capable of direct hormonal modification. However, direct response to pharmacological quantities of insulin need not connote physiological significance nor justify extrapolations concerning the contribution of insulin to the normal function of that tissue in the intact animal. Such conclusions require the demonstration of metabolic defects in the "insulin-responsive" tissue following withdrawal of insulin *in vivo*.

Accordingly, to fulfill this second criterion, pituitaries from animals with experimental diabetes were examined. Carbohydrate metabolism was reduced 30 to 50 per cent below control values in anterior pituitaries derived from rats rendered acutely aninsulinemic by total pancreatectomy. The magnitude of this derangement approaches the degree of impaired glucose utilization which has been observed in such classically insulin-responsive tissues as diaphragm (37, 38) or adipose tissue (39) from diabetic rats. Furthermore, the defect correlated best with the degree of insulin lack rather than of operative trauma. Thus, it was not observed in partially pancreatectomized animals or in rats with surgically induced shock or hypovolemia in which pancreatic structure was preserved. Moreover, in the alloxanized animals in which absolute insulin deficiency was not achieved (as judged by their limited inanition and their survival in the untreated state), reduction of pituitary carbohydrate metabolism was not as profound. It would seem unlikely that the hypophyseal "biochemical lesions" in the diabetic animals represented merely secondary consequences of ketonemia, acidosis or lipemia, since significant differences could not be demonstrated between animals in which total pancreatectomy was uncomplicated, and animals in which circulatory collapse precluded the full development of post-pancreatectomy ketogenesis (25) or lipid mobilization. Indeed, the manifest association between insulin deprivation *in vivo* and deterioration of pituitary carbohydrate metabolism reinforces the significance of the small effects which were obtained by directly adding insulin to normal rat AP *in vitro*.

Theoretically, for any given tissue, the third criterion of physiological insulin-responsiveness should consist of the correction of metabolic defects by the *in vivo* administration of insulin to insulin-deficient animals. In the present studies with anterior pituitary, this objective was also fulfilled. Exhibition of exogenous insulin within hours following total pancreatectomy completely prevented the decline of hypophyseal carbohydrate economy. Moreover, in animals with chronic hypoinsulinism, i.e., the alloxanized rat, therapy with insulin enhanced the metabolic performance of pituitary tissue. Although precise temporal relationships were not fully documented, the reversibility would indicate that permanent alterations in the carbohydrate metabolism of the anterior pituitary did not result from 6 weeks of partial insulin deprivation.

Thus, by the most rigid criteria, it would appear that the anterior pituitary must be included in the growing list of insulin-responsive tissues (40). Indeed, the present demonstration that intrahypophyseal utilization of glucose will respond to insulin *in vitro*, deteriorates following insulin withdrawal *in vivo*, and can be restored by insulin administration to insulin-deficient animals *in vivo*, would suggest that the anterior pituitary must be considered "diabetic" in the syndrome of diabetes mellitus. For the moment, translation of the findings into more definitive implications for normal homeostasis within the pituitary or pathological aberrations in spontaneous diabetes mellitus must be deferred. First, since the cellular population of the anterior pituitary is not homogeneous, it remains to be defined which cell types are responsive to insulin. Possibly, only a limited proportion might be affected or response-thresholds of various elements might differ considerably. That some cellular components may be altered is suggested by the reported occurrence of basophilic changes in the pituitaries of totally pancreatectomized dogs (41). Second, the precise relationship between hypophyseal carbohydrate metabolism and intrapituitary turnover of hormones remains to be elucidated. By analogy to other systems (42–44), the pathways which have been described in the present studies for the storage of glucose, the mobilization of glycogen, and the oxidation of glucose-6-phosphate within the pituitary could subserve important functions in selective or generalized hormonogenesis. Derangement of these pathways and diminished energy generation through impaired oxidation of glucose might not only alter the balanced biosynthesis of known pituitary hormones, but could even promote the formation of abnormal, biologically potent protein or peptide principles. However, elaboration of anterior pituitary hormones also entails storage, release, and responsiveness to peripheral as well as to suprahypophyseal regulators. It is conceivable (45) that any or all of these processes might be conditioned by pituitary carbohydrate metabolism.

At present, there seems to be no compelling

clinical evidence for derangement of pituitary function in spontaneous diabetes mellitus. On the other hand, the relatively crude methods which have been available for such evaluation do not constitute compelling reasons for discarding the possibility. The present studies with laboratory animals highlight the critical need for re-examination of the possible interrelationships and afford a feasible experimental approach for this type of inquiry.

SUMMARY

1. Pathways of carbohydrate metabolism have been examined in surviving preparations of normal calf and rat anterior pituitary. Mechanisms for active glycogen turnover and glucose oxidation via the hexose-monophosphate shunt *in vitro* have been demonstrated. Anterior pituitaries from both species responded to the direct addition of insulin to the suspending media.

2. Micro-methods have been developed for incubation of single rat anterior pituitaries. With this technique, it has been demonstrated that hypophyseal carbohydrate metabolism is significantly reduced in anterior pituitaries excised from rats rendered acutely insulin-deficient by total pancreatectomy or chronically diabetic by alloxanization. The phenomenon could not be attributed to such systemic manifestations of insulin deprivation as ketoacidosis and could not be duplicated by comparable degrees of surgical trauma in animals in which pancreatic function was preserved. The derangement of hypophyseal glucose utilization in rats with experimental diabetes could be prevented or reversed by treatment with replacement quantities of insulin *in vivo*.

3. The data indicate that the anterior pituitary is responsive to insulin and that intrahypophyseal carbohydrate metabolism in the intact animal may be conditioned by the availability of insulin. The significance of insulin dependency in terms of the normal endocrine function of the anterior pituitary and pituitary status in spontaneous diabetes mellitus remains to be elucidated.

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LUNG AND CHEST WALL COMPLIANCE OF APNEIC PARALYZED INFANTS *

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The total respiratory compliance of anesthetized, relaxed adults has been found to have a lower value than that in awake subjects (1, 2). The probable explanation for this fact is the inability of the conscious individual to relax as completely as does the anesthetized patient. The values observed in unconscious subjects, therefore, more closely approximate those of narcotized patients in need of artificial respiration (1, 2) or of unanesthetized, apneic polio patients being ventilated by a mechanical respirator (3).

The pulmonary compliance of resting newborn infants has been measured by the use of a body plethysmograph and intraesophageal pressure technique (4, 5). These values are low when compared on a lung weight basis to those recorded for the adult. However, since lung compliance relates closely to the functional residual capacity (4, 6, 7), a comparison of infant and adult lungs on this basis suggests that they are similar in compliance.

We have been unable to find any report of measurements of total lung and chest wall compliance in the anesthetized, paralyzed infant. This is a report of our endeavor to obtain such information, which, we believe, will provide a better understanding of infant respiratory physiology. These data also are of practical interest for infant resuscitation, anesthesia, and in the design of infant resuscitators.

METHODS

The 21 infants selected for this study varied in age from 5 to 76 days and in weight from 5.5 to 12.5 pounds. Three of the infants were females. All appeared free of respiratory disease as far as could be determined by history and physical examination. None exhibited abdominal distention. All were scheduled for pyloroplasty or inguinal hernioplasty, except one who was to have a liver biopsy and one excision of a sacral cyst. Three infants were premature at birth.

The apparatus (Figure 1) consisted of an oxygen cylinder with pressure-reducing valve and flow-regulator (a flow-regulating valve designed to deliver gas constantly at a selected pressure), a large-bore two-way stopcock and a calibrated, low-inertia water spirometer of approximately 450 ml capacity. These were connected by large-bore, smooth rubber tubing to permit inflation of the infant's lungs with oxygen through one limb of the two-way stopcock, then rapid collection of the passively expired gas in the spirometer through the other limb. The "compliance" of the rubber components of the equipment was negligible at the highest pressure.

All infants were studied in the operating room just prior to operation. Premedication consisted of atropine sulfate, 0.15 mg, given intramuscularly 38 to 126 minutes before the start of anesthesia. The infants were placed in a supine position on the operating table, and were then allowed to inhale a mixture of 40 per cent cyclopropane in oxygen via a face mask and bag. Anesthetic inhalation continued until the infant stopped moving and appeared to lose consciousness, usually 1 to 2 minutes (3 minutes for one infant and 4 minutes for another). At this point, succinylcholine chloride, 2.0 to 3.8 mg per

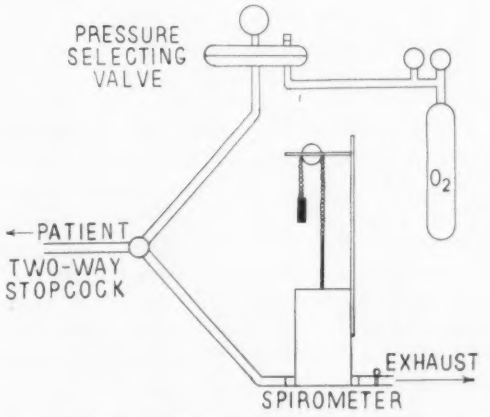

FIG. 1. DIAGRAM OF APPARATUS USED FOR TOTAL COMPLIANCE MEASUREMENTS. The infant's lungs were inflated for 10 seconds at the selected airway pressure, then the stopcock was turned rapidly to collect the passively expired gas in the spirometer.

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pound of body weight, was administered intramuscularly and the cyclopropane was turned off. One infant required two doses of succinylcholine, totaling 5.8 mg per pound of body weight. As apnea developed, usually within 1 to 3 minutes, the infant's respirations were gently assisted, then controlled, with oxygen. Endotracheal intubation was accomplished with a close-fitting tube within 5 minutes of the injection of succinylcholine. Immediately thereafter, a nasogastric catheter was passed to assure deflation of the stomach while the lungs were gently ventilated with oxygen. Compliance measurements were commenced within 1 to 2 minutes after the tracheal intubation, and each study required less than 5 minutes to complete, during which time respiratory activity returned in only one infant. In view of the brief duration of cyclopropane administration, and the 3 to 6 minutes of washout with oxygen before beginning the study, the concentration of cyclopropane in the infant during the measurements was considered to be negligible.

The procedure consisted of inflating the infant's lungs with oxygen for 10 seconds at five different airway pressures and collecting the passively expired oxygen in the spirometer after each inflation. The infant's lungs thus deflated to their resting expiratory level after each volume

TABLE I

Physical characteristics, succinylcholine dosage and total respiratory compliance data for 21 normal infants

Patient no.	Age	Wt	Ht	Dose of succinylcholine	Total compliance (*ml/cm H_2O*) At 6.8–8.2 cm H_2O	At 13.6 cm H_2O	At 20.4 cm H_2O	At 27.2 cm H_2O	At 30.0–31.2 cm H_2O	At 32.6–34.0 cm H_2O	Series order
	days	*lbs, oz*	*cm*	*mg/lb*							
1*	43	5,8	49	3.3	3.3	5.1	4.9	4.4		4.1	a. Descending
					3.3	3.3	3.6	3.9		3.9	b. Ascending
2	5	6,0	47	3.0	4.7 †	5.0	5.1	5.0		4.5‡	a. Descending
					4.0 †	4.5	4.9	4.9		4.9‡	b. Ascending
3	44	6,10	51	3.0	6.5	6.6	5.7	5.1		4.8	a. Descending
					6.3	6.2	5.4	4.9		4.6	b. Ascending
4	34	6,11	52	3.0	5.6	6.1	5.3	4.6	4.6§		a. Descending
					5.6	6.1	4.8	4.7	4.5§		b. Ascending
5	46	6,12		2.1	7.1	5.5	4.7	4.5	4.6§		b. Ascending
					7.1	7.0	5.7	5.1	5.2		a. Ascending
6	23	7,0	50	3.6	6.5	7.3	6.2	5.7		5.1	a. Descending
					6.5	7.0	6.2	5.7		5.1	b. Ascending
7	46	7,6	52	3.4	7.4	7.0	6.5	5.3		5.1‡	a. Descending
					7.4	7.0	6.0	5.1		5.1‡	b. Ascending
8‖	20	7,12	55	3.8	5.7	7.0	5.7	5.1		4.6	a. Descending
					5.7	6.2	5.4	5.1		4.7	b. Ascending
9	45	8,4		2.2	5.6	5.7	4.7	4.3	4.4		b. Ascending
					(5.1)†	(5.5)	(4.4)	(Breathing)			a. (Not incl.)
10‖	26	8,4		2.0	3.4 †	3.1	2.6	2.9		4.4‡	b. Ascending
					5.1	5.1	4.4	4.3	4.8		a. Ascending
11	28	8,4	54	2.0	6.5	7.0	5.7	4.9		4.4‡	a. Descending
					6.2	6.6	5.5	4.9		4.6	b. Ascending
12‖	40	8,14	49	3.4	3.2	4.6	4.1	3.6		3.7	a. Descending
					3.2	4.1	3.6	3.6		3.7	b. Ascending
13*	55	9,1	52	3.3	4.1	4.3	3.9	3.7		3.6‡	a. Descending
					3.3	3.9	3.4	3.5		3.9‡	b. Ascending
14*	76	9,4	54	2.7	4.1	5.9	4.9	4.3		3.9‡	a. Descending
					4.1	5.1	4.2	3.9		4.1‡	b. Ascending
15	58	9,15		2.5	4.0 †	4.6	4.1	4.6	5.8		b. Ascending
					5.4 †	6.9	5.9	5.7		5.7‡	a. Ascending
16	43	10,12	57	2.3	4.8	6.6	5.4	4.9		4.9‡	a. Descending
					4.8	5.5	4.7	5.1		5.1‡	b. Ascending
17	28	11,4	52	3.2 / 2.6 = 5.8	4.1	5.9	5.3	4.9		4.7	a. Descending
					4.1	4.6	4.1	4.5		4.9	b. Ascending
18	64	11,8	57	2.6	4.1 †	4.7	4.5	4.7		5.8‡	b. Ascending
					5.0	6.2	5.8	5.5		5.8‡	a. Ascending
19	51	11,10	57	2.6	4.8 †	6.7	6.1	5.6		5.2‡	a. Descending
					4.1 †	5.6	5.0	5.0		5.5‡	b. Ascending
20	40	11,12	58	3.1	5.6	7.6	7.0	6.2		5.7	a. Descending
					5.6	6.9	5.9	6.0		5.8	b. Ascending
21	46	12,8	61	3.2	4.1	5.9	5.1	4.5		4.2	a. Descending
					4.1	4.6	4.1	4.1		4.2	b. Ascending
Average					5.2	6.2	5.4	4.9	4.9	4.7	a. Descending or second ascending
SD					±1.0	±0.9	±0.9	±0.8	±0.4	±0.6	
Average					4.9	5.2	4.6	4.6	4.8	4.7	b. Ascending
SD					±1.2	±1.4	±1.0	±0.4	±0.8	±0.8	

* Premature at birth.
† Measured at 8.2 cm H_2O.
‡ Measured at 32.6 cm H_2O.
§ Measured at 30.0 cm H_2O.
‖ Female; all others were male.

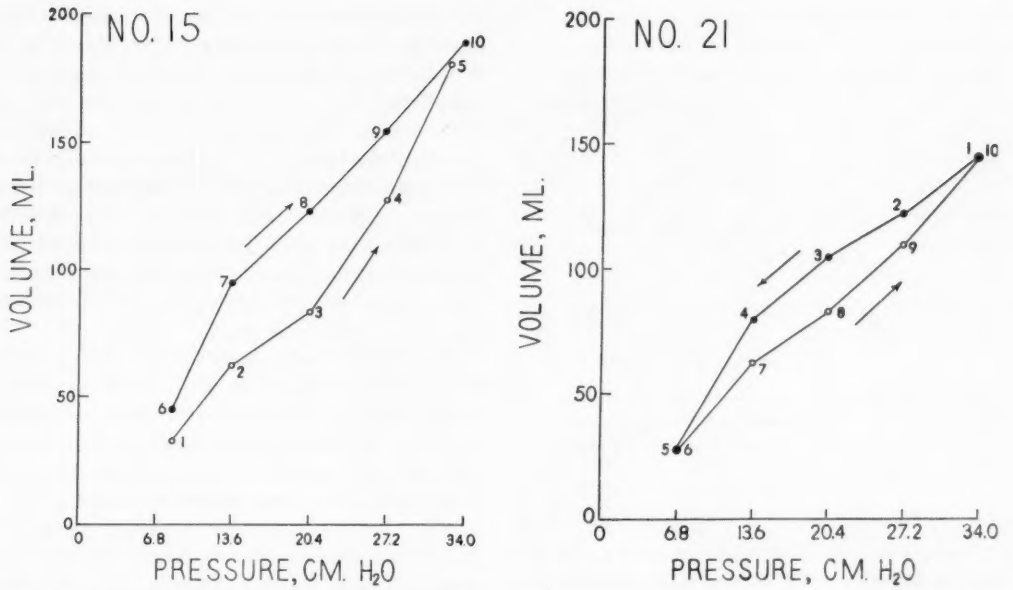

FIG. 2. REPRESENTATIVE PRESSURE-VOLUME CURVES. The volume measurements are numbered in the sequential order of study. The effect of a previously higher airway pressure on a subsequent measurement is apparent for each infant in the differences between the curves.

measurement and were reinflated from this level during the succeeding application of pressure. The endotracheal tube fit snugly enough in the larynx to prevent gas leaks at the higher airway pressures. Spirometer collection times at each pressure were about 4 to 6 seconds, ample time to insure no further gas flow from the lungs. Expired volumes were corrected to BTPS.

Two volume measurements were made at each airway pressure. Five infants were studied, starting at the lowest pressure and progressing to the highest pressure, then repeating the series in the same order. The remainder of the infants were studied by starting at the highest pressure and progressing to the lowest pressure, then repeating the series in the reverse order. The pressures chosen were 6.8, 13.6, 20.4, 27.2 and 34.0 cm H_2O; these correspond to 5, 10, 15, 20 and 25 mm Hg, respectively. A few measurements were made at 8.2, 30.0, 31.2 or 32.6 cm H_2O.

RESULTS

Compliance values for the lungs and thorax at each airway pressure are listed in Table I. An individual value for compliance represents the ratio of the volume expired to the airway pressure applied immediately preceding the expiration. Compliance was noted to be higher when measurements were made starting at a high pressure and progressing to a low pressure, or when a second ascending series of pressures followed immediately after the highest pressure of the previous ascending series.

Two representative pressure-volume curves are shown (Figure 2). The volume measurements are numbered in the sequential order of study; thus, in Infant 15 both studies were ascending in order, and in Infant 21 the first study was descending in order and the second was ascending.

The compliance averages at the various pressures for each type of series, together with the standard deviation of compliance values, are presented at the bottom of Table I. These averages have been plotted graphically against the airway pressures used (Figure 3). The fact that higher compliance values resulted when measurements were made following a higher airway pressure is clearly demonstrated by the differences between the curves. These differences were most marked at pressures of 13.6 and 20.4 cm H_2O ($p < 0.01$ and $p < 0.02$, respectively [1]). The curves approached one another at the pressure extremes.

Comparisons of data from individual infants can be made at the compliance values obtained at 20.4 cm H_2O of the descending or second ascending

[1] Difference equals 2.7 and 2.5 times the standard error, respectively.

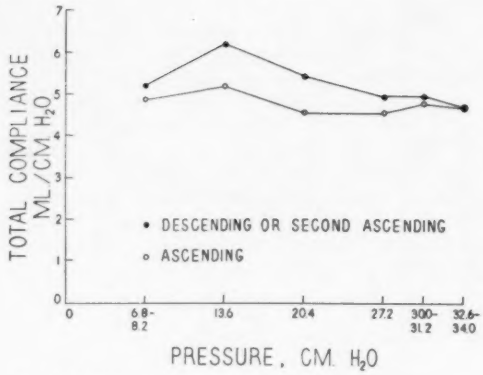

FIG. 3. TOTAL COMPLIANCE AVERAGES AT VARIOUS AIRWAY PRESSURES. Compliance is greater at a given pressure if the measurement is made following an inflation at a higher airway pressure.

series of measurements. This is an arbitrary comparison point used by other investigators (1) and selected here for two reasons. It was the midpoint of the pressure range used and, therefore, should be less affected by variables which might appear at either extreme of the pressure range. Also, there was a sufficiently wide range of values at this pressure to show correlation between compliance and physical characteristics, if such existed. There appeared to be no correlation between the total compliance at this pressure and the infant's age, weight or height.

DISCUSSION

The purpose of these experiments was to measure the total lung and chest wall compliance of infants, a facet of infant respiratory physiology previously not reported. In order to eliminate muscular effort, large doses of a neuromuscular blocking agent were employed.

Data are available to suggest that true compliance values can be obtained only if the volume of inflation is measured after the lungs and chest wall have been permitted to adjust to the applied pressure for 5 or more seconds. Butler and Smith (2) noted in adults that airway pressure would decline shortly after the lungs were distended with a given volume increment. This they attributed to the gradual opening of collapsed segments on the lung surface, with a consequent increase of effective alveolar volume. Airway pressure stabilized after 2 or 3 seconds. High intrapulmonary pressure in the supine adult also may result in the displacement of an appreciable amount of blood out of the thorax, which would lead to an increase in compliance (1). Similar effects might be expected in the infant. In order to minimize or eliminate variables which might result from an airway pressure of short duration, such as differences in tissue friction, resistance to movement, alveolar cohesiveness, pulmonary blood volume, and collapsed segments on the lung surface, we have recorded each volume measurement after chest inflation had been maintained for 10 seconds.

Pulmonary compliance values depend upon whether measurements are made from the normal resting volume or following a deep inspiration. Similarly, total compliance values vary depending upon whether positive pressure inflation is applied at the thoracic resting volume or following a previously greater expansion of the lungs and thorax. Butler (8) observed that the lungs and chest wall of adults, upon passive deflation, do not return immediately to their initial resting values after inflation to 20 cm H_2O pressure. During chest expansion, relaxed muscles stretch and abdominal contents are displaced downward. Relaxed muscle which has undergone distortion does not regain at once its original length when tension is released. In addition, frictional and surface tension forces oppose the return of displaced abdominal contents and result in a time-dependent deformation after inflation. A return to resting values occurs after about 10 seconds. In this study, usually less than 10 seconds elapsed between complete passive deflation and subsequent inflation. Consequently, volume measurements in the descending and second ascending series probably were affected somewhat by the residual distortion of the lung, chest wall and abdominal contents from the preceding higher pressure.

Total compliance values are greater if one progresses from the high pressure-volume recordings toward the lower ones, rather than in the opposite direction. Artifacts consequent to surface tension and lung blood volume are reduced by this technique, thereby affording more reliable data. The pressure-volume curves obtained in this study resemble those reported by Agostoni (9) for anesthetized, paralyzed newborn dogs. The total compliance of these newborn dogs was low over the tidal volume range during the initial ascending

series of pressure-volume measurements, but a marked increase in the slope of the curve occurred at higher inflation pressures, indicating the opening of previously nonaerated alveoli. After a full expansion was over, compliance values were greater in the subsequent pressure-volume measurement, implying that most of these alveoli remained open.

Several investigators (4, 6, 7) have concluded that pulmonary compliance is related to functional residual capacity, which in turn is related to an exponential function of body height (6). Although in our study there was a range of 71 days in age, 7 pounds in body weight, and 14 cm in height, no significant correlation could be found between the total compliance value at 20.4 cm H_2O and age, weight or height. Possibly the number of infants studied was too small to reveal such a correlation.

Pulmonary compliance in newborn infants has been measured by others (4, 5). Our data suggest that the pulmonary and total compliance of the supine infant are nearly the same. Cook and his associates (4) found an average pulmonary compliance of 5.2 ml per cm H_2O during quiet respiration in 18 infants aged 1 hour to 7 days. Swyer, Reiman and Wright (5) measured a pulmonary compliance of 4.9 ml per cm H_2O in 15 sleeping infants aged 2 hours to 11 days. In both studies the values were calculated from the ratio of tidal volume to the change in intraesophageal pressure measured between points of no flow, i.e., at the extremes of tidal volume. Although in both of these groups the infants were younger than those in our study, we have thought it worthwhile to compare their results for pulmonary compliance and ours for total compliance. For this purpose, 6.8 cm H_2O positive airway pressure most closely approximates the negative change in intraesophageal pressure observed by the above two groups. At this airway pressure, our average total compliance was 5.2 ml per cm H_2O when immediately preceded by a higher pressure, and 4.9 ml per cm H_2O when not immediately preceded by a higher pressure. These values approximate closely those reported for the infant's pulmonary compliance. This indicates that the compliance for the thorax, alone, is high in infants. A high chest wall compliance has been measured in newborn dogs (9).

The influence of the diaphragm and abdominal contents on respiratory expansion in the supine infant is presumed to be about the same whether the infant is breathing spontaneously or by positive pressure inflation in the presence of apnea. The major factor to overcome in either case is the hindrance of diaphragmatic excursion by the inertia and frictional resistances of the abdominal contents. Therefore, the similarity between pulmonary and total compliance in the infant implies that the infant's thorax has little influence on pulmonary expansion because of its characteristic anatomical structure (i.e., horizontally placed ribs and cylindrical shape).

CASE PRESENTATION

Decreased pulmonary compliances have been reported in infants with respiratory disease (4, 10, 11). The following case report describes an infant demonstrating decreased total compliance 12 hours before signs of respiratory disease became evident.

This 7 pound 4 ounce male, with an imperforate anus, was admitted to this hospital within 8 hours of his birth. There was no evidence of pulmonary disease or abdominal distention by history, physical examination or roentgenogram. An anoplasty was performed when the infant was approximately 24 hours old. Atropine, 0.15 mg, was given intramuscularly 30 minutes prior to induction of anesthesia with cyclopropane and oxygen. The technique of anesthetic induction and tracheal intubation was by the method described for compliance studies. Succinylcholine, 6.0 mg per pound of body weight, was given intramuscularly in two doses.

Compliance measurements were made within 4 minutes of the first injection of muscle relaxant. The pressures used were 6.8, 13.6, 20.4, 27.2 and 34.0 cm H_2O, starting and ending at the highest pressure. The compliance values for the descending series were 3.1, 3.1, 3.5, 3.2 and 2.9 ml per cm H_2O; the values for the ascending series were 2.9, 3.1, 3.0, 3.1 and 2.9 ml per cm H_2O. The reason for the decreased compliance was not immediately evident.

Anesthesia and operation were continued without complications, and the postoperative course seemed normal for the first 12 hours. At this time the infant's respirations became labored, his chest retracted with each breath, and copious respiratory secretions developed. This respiratory distress progressively became worse over the next 12 to 20 hours, and several laryngoscopic tracheal aspirations were necessary to remove the thick secretions. It was feared that this represented hyaline membrane disease, but a chest roentgenogram was interpreted as "slight pulmonary hyperaeration." By 36 hours after operation, and following antibiotic therapy and aspiration of respiratory tract secretions, the infant's symptoms began to subside, and by 48 hours after operation, his condition was improved markedly.

Whatever the cause of this infant's respiratory prob-

lem, we were able to demonstrate that the total compliance was decreased appreciably 12 hours before the onset of clinical respiratory symptoms.

SUMMARY

Measurements of the total lung and chest wall compliance at various airway pressures in 21 normal infants paralyzed with succinylcholine have been reported. Data are presented which show that the average total compliance for a given airway pressure is greater when immediately preceded by an inflation at a higher airway pressure than by one at a lower airway pressure. The similarity between values for pulmonary and total compliance in the infant is noted and discussed. In addition, a case has been presented in which a decreased total compliance was measured in an infant 12 hours before signs of respiratory disease became evident.

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A METHOD FOR DETERMINING INERT GAS ("N_2") SOLUBILITY IN URINE *

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The present interest in the physiology of inert gases makes desirable a more accurate and reproducible method for determining the inert gas content of biological fluids than is currently available (2). This communication describes such a method. The method has been tested on water and used to determine the solubility of the inert gas of air in human urines of different solute concentrations at 37° C.[1] Empirical relationships of solubility to osmolality, chloride concentration, and specific gravity are given. Preliminary studies indicate that the method can also be applied to blood. Another communication (3) will describe the method's application to human urine in a manner believed to enable the calculation of the inert gas tension of arterial blood within ± 2 mm Hg.

METHOD

The method to be described is similar to one employed by Van Slyke, Dillon and Margaria (4). It consists essentially in determining the total content of gas other than CO_2 and O_2 in a 25 ml sample of fluid, using a Van Slyke manometric apparatus. This gas will be referred to as nonabsorbable gas. A more complete description and discussion of the method than can be given here appears elsewhere (5). The basic procedure is outlined below.

Special reagents and apparatus. 1) CO_2–O_2 absorber. Twenty g of sodium hyposulfite ($Na_2S_2O_4$) is dissolved in 100 ml 3 N KOH and filtered rapidly through gauze to remove any insoluble impurity. Three N KOH was chosen arbitrarily because it provides enough hydroxide to ensure alkalinity of any 25 ml urine sample. No sodium anthraquinone-B-sulfonate is included because the red color produced makes critical adjustment of the fluid meniscus to the 0.5 ml mark more difficult. *2) Ostwald-Van Slyke pipets.* Each pipet was equipped with rubber tip and stopcock and custom-built to deliver 25 ml.

Evacuation of reagent and introduction of sample. Three to four drops of caprylic alcohol and CO_2–O_2 absorber to make 7 ml are drawn into the Van Slyke extraction chamber and doubly extracted (2 minutes per extraction). Five ml of the alcohol-absorber reagent is left in the extraction chamber and the bore of the stopcock leading to it after the second extraction. The sample is admitted from the Ostwald-Van Slyke pipet, with the rubber tip of the pipet sitting tightly at the bottom of the cup. A drop or two of Hg is added to the cup to prevent any of the excess reagent from entering the extraction chamber. Great care is taken to prevent any small air bubbles from entering the chamber with the sample. Bubbles frequently lodge at the pipet stopcock. Their number may be minimized by having the stopcock and adjacent pipet bore scrupulously clean and by manipulating the stopcock under visual observation during filling. This manipulation is easier when pipets are filled in an "inverted" fashion; in addition, dislodged bubbles do not pass through the 25 ml sample. Bubbles also lodge at the rubber tip of the pipet. Their number may be minimized by not adjusting the meniscus to the upper calibration mark until just before the sample is introduced, by keeping the pipet vertical after this adjustment, and by not dislodging any small drop of sample extending below the rubber tip.

Extraction and reading. The Hg in the extraction chamber is brought to the 50 ml mark and the reaction mixture (reagent + sample) is shaken vigorously for 8 minutes (by magnetic stirrer). Care is taken to readjust the Hg to the 50 ml mark during the first 1 to 2 minutes. The fluid meniscus in the chamber is brought to the 0.5 ml mark and a manometer reading is taken. The extraction process is repeated for 3 minutes, and another manometer reading is taken at the 0.5 ml mark. The Hg is returned to the 50 ml mark and kept there for 45 minutes without shaking, except for three manometer readings at the 0.5 ml mark at 15, 30 and 45 minutes. At the 45 minute reading, which is designated P_1, the temperature of the water jacket surrounding the extraction chamber is recorded to the nearest 0.1° C. After reading P_1, another 3 minute extraction is given, fol-

* A preliminary report involving this work was presented at the April, 1959 meetings of the Federation of American Societies for Experimental Biology, Atlantic City, N. J. (1). This study was supported in part by the Air Research and Development Command, Wright-Patterson Air Force Base, Ohio.

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[1] Since the inert gas of air is often treated as being entirely N_2, its solubility in a fluid has been referred to as "atmospheric nitrogen" or "N_2" solubility.

lowed by another manometer reading at the 0.5 ml mark. The extracted gas is then ejected quantitatively without loss of any reaction mixture, as described by Peters and Van Slyke (6). The fluid meniscus is again brought to the 0.5 ml mark and manometer reading P_2 is taken. The analysis is then complete.

A definitive P_1 reading can be made only under three conditions: when there has been complete absorption of CO_2 and O_2 and partial pressure equilibrium of the nonabsorbable gas between the gas and liquid phases, and when the thermometer in the water jacket represents the true temperature within the extraction chamber. The initial 8 and 3 minute shaking periods are intended to satisfy the first two conditions; the agreement of the first two manometer readings indicates that this is the case. The further serial readings at 15-minute intervals are believed necessary to satisfy the third condition. Many factors can raise the temperature within the extraction chamber above its pre-analysis level. Other factors suggest that the secondary increase in water jacket temperature will be delayed, smaller and more protracted; of great importance may be the unfavorable ratio of fluid surface to volume when 30 ml of reaction mixture is contained in a 50 ml extraction chamber. This situation is proposed as the explanation for changes in manometer readings and water jacket temperatures we have regularly observed. The manometer reading decreases a few millimeters of Hg during the first 15 to 25 minutes of the 45 minute waiting period. The water jacket temperature increases ($\sim 1°$ C) during the 11 minutes of shaking and during the first 5 to 15 minutes of the 45 minute waiting period; it then slowly returns to room temperature. The importance of this temperature problem was alluded to by Peters and Van Slyke (6) several years ago. With the method reported, P_1 and the reading preceding it almost always agree within 1 mm Hg (an error of only 0.0015 ml in setting the meniscus at the 0.5 ml mark could account for such a 1 mm pressure difference). The final 3 minute extraction period is included to be sure no nonabsorbable gas has been redissolved into the fluid phase whenever the volume of the gas phase has been reduced from 20 to 0.5 ml.

Calculations. The general equation for the gas content (V) of a fluid sample analyzed with a manometric aparatus has been developed by Van Slyke, Neill and Stadie (7, 8). With the method reported, four factors in this equation become constants ($a = 0.5$ ml, $S = 30$ ml, $A = 50$ ml and $s = 25$ ml). P, the observed partial pressure (in millimeters of Hg) exerted by the gas when reduced to fixed volume at known temperature, is taken as $(P_1 - P_2)$. No "c" (blank analysis) correction is required (7). The original Van Slyke equation therefore simplifies to:

$$V = (P_1 - P_2) \times \frac{0.002632\ i}{(1 + 0.00384\ t)} (1 + 1.50\ \alpha') \quad [1]$$

where V = nonabsorbable gas content (volumes per cent, STPD); i = experimental correction factor for reabsorption of gas from gas into fluid phase when volume of gas phase is reduced to 0.5 ml; t = temperature (°C) of water jacket at time of reading P_1; α' = the ratio in which the nonabsorbable gas mixture being analyzed distributes itself between equal volumes of the gas and fluid phases when extraction is complete (Ostwald coefficient). The factor, i, is dependent upon several other factors, the most important of which is the solubility of the gas mixture being measured in the reaction mixture being used. Determinations of i for common gases have been described (7, 8); α' can be calculated for any given gas mixture and temperature (8, 9).

EVALUATION OF METHOD ON EQUILIBRATED WATER

The accuracy and reproducibility of the method were tested by analyzing 46 successive distilled water samples equilibrated with compressed air at 37° C.

Equilibration technique. Several of the equilibration techniques investigated proved unacceptable. For example, when compressed air is simply bubbled through a column of water, the total pressure in each bubble exceeds P_B because of the hydrostatic pressure. The amount of this excess pressure varies with the depth and size of the bubble and is difficult to determine. The exact value of P_{H_2O} may also be uncertain, since there is no assurance that each bubble is 100 per cent saturated. While these considerations are usually unimportant in the equilibration of fluids with CO_2 and O_2 in their physiological ranges, they are extremely important for N_2. Every 1.2 mm error in water vapor tension or total gas pressure causes a 1 mm error in the usual calculation of inert gas tension. The increased inert gas constant resulting from this equilibration technique is sufficient to be detected by the method reported.

The modification of the spherical tonometer of Laue, described by Finley and co-workers (10), was finally adapted to obtain rapid, accurate equilibration of the water. The only change was the omission of the syringe used to withdraw the equilibrated fluid; the center arm of the distilling flask was instead occluded with a rubber stopper. A minimal continuous flow of saturated gas was maintained from a tank of compressed air. Distilled water, 35 to 50 ml (containing less than 0.2 parts per million NaCl) was added to the flask and swirled 30 minutes, the speed of rotation being adjusted so that the water formed a thin film more than half-way up the walls of the flask. The pressure within the flask never exceeded atmospheric by more than 0.5 cm H_2O. The

temperature of the water bath did not vary more than $\pm 0.1°$ C.

Equilibrium was found to be approached logarithmically by analyses of inert gas-free water kept in a tonometer for various periods of time. Ninety per cent of equilibrium content was reached in 3 minutes and 99 per cent in 6 minutes. To transfer the equilibrated water to an Ostwald-Van Slyke pipet, the rubber stopper was removed and the long upper stem of the pipet inserted to the bottom of the distilling flask. The pipet was thus filled in the inverted fashion previously described. It is interesting that no change in nonabsorbable gas content could be detected after a filled pipet had been allowed to stand at room temperature for as long as 8 hours.

Predicted inert gas content. Since the amount of gas physically dissolved in a fluid is the product of its partial pressure and its solubility at the temperature of the fluid, the predicted inert gas content of any sample was given by the following equation. In order to simplify the nomenclature, the usual subscript N_2 (designating the N_2-A mixture that is the inert gas of air) will be omitted.

$$V_P = \frac{\alpha_{w_{37}}}{760} \times F_I(P_B - 47.1) \times 100 \quad [2]$$

where V_P = predicted inert gas content (volumes per cent, STPD); $\alpha_{w_{37}}$ = volume of the inert gas of air (STP) absorbed by 1 vol of distilled water at 37° C when the dry pressure of the gas is 760 mm Hg (Bunsen coefficient); F_I = inert gas fraction in the compressed air used for equilibration; and P_B = ambient pressure (mm Hg) during equilibration. The inert gas of the compressed air was assumed to be 98.8 per cent N_2 and 1.2 per cent A (11); $\alpha_{w_{37}}$ was calculated to be 0.01244, using this equation:

$$\alpha_{w_{37}} = 0.988\alpha_{w_{N_{237}}} + 0.012\alpha_{w_{A37}}. \quad [3]$$

Values for $\alpha_{w_{N37}}$ and $\alpha_{w_{A37}}$ were obtained by interpolating values in standard tables (12–14). A study of the literature (4, 13–16), showed that the values for N_2 solubility in standard tables represent N_2 solubility alone, and not the solubility of the N_2-A mixture found as the inert gas of atmospheric air. F_I was checked with a Scholander gas analyzer (17), and always taken as 0.7903 (11). P_B varied from 733.6 to 749.4.

Determined nonabsorbable gas content. The nonabsorbable gas content, V_d (volumes per cent, STPD), of each water sample was measured with the method reported, using one of three extraction chambers. The nonabsorbable gas measured was presumably 97.5 per cent N_2 and 2.5 per cent A, since A is over twice as soluble in water as N_2. In applying Equation 1, i was always taken to be 1.000 (4); α' values were obtained from the usual equation (8, 9) after determining the appropriate Bunsen coefficients for a 97.5 per cent N_2-2.5 per cent A mixture in water (see above). This assumed that the solubility of nonabsorbable gas in the reaction mixture was the same as that in water. Since it is actually less than in water (7), α' has in each case been overestimated by a small but definite constant amount. Temperatures of analyses varied between 24.4 and 28.8° C.

Composition of nonabsorbable gas. The question arises as to whether the "nonabsorbable gas" in the extraction chamber at the time of reading P_1 was entirely N_2 and A, in the ratio expected from their relative concentrations in air and solubilities in water. The composition of the gas has therefore been checked by mass spectrometer analysis. To collect the gas, the stopcock at the top of an extraction chamber was replaced with a high vacuum stopcock. Distilled water equilibrated with compressed air at 37° C was then analyzed in the usual fashion. After reading P_1, the nonabsorbable gas was ejected (through an all glass evacuated system) into a special mass spectrometer sampling tube equipped with a high vacuum stopcock. The nonabsorbable gas from four successive water analyses was collected in one sampling tube and found to have the composition shown in Table I. The sample was scanned from mass 2 to mass 200.

TABLE I

*Mass spectrometer analyses of nonabsorbable gas in Van Slyke extraction chamber at time of reading P_1**

Specimen	N_2	A	CO_2	O_2	"Hydrocarbon"
	%	%	%	%	%
Equilibrated water	97.4	2.3	0.11		0.15
Equilibrated urine (lab worker)	98.0	1.9	0.04		0.03
Equilibrated urine (emphysema patient)	97.1	2.4	0.33	0.06	0.06

* Results have been calculated on a water-free basis.

TABLE II

Comparison of predicted inert (V_p) and determined nonabsorbable (V_d) gas content of 46 successive distilled water samples equilibrated with air at 37° C

Extraction chamber	V_p/V_d	
	Mean	SD*
1 (n = 14)	0.9904	0.004
2 (n = 21)	0.9976	0.003
3 (n = 11)	0.9897	0.002

* Standard deviation.

It is significant that 99.7 per cent of the nonabsorbable gas was N_2 and A. There was no significant difference between the observed N_2 to A ratio (97.4/2.3) and the predicted N_2 to A ratio (97.5/2.5). There was no mass 12 peak to indicate that any of the "N_2" measured was CO. The 0.15 per cent "hydrocarbon" has not been identified specifically.

Discussion. The ratio of V_p to V_d was determined for all 46 analyses. The results are summarized in Table II. The ratios consistently fall 0.2 to 1.0 per cent below unity, i.e., determined nonabsorbable gas contents regularly exceed predicted inert gas contents by 0.2 to 1.0 per cent. This constant "excess" nonabsorbable gas is at least partly due to the overestimation of α' already described. It could conceivably also result from repeated setting of the fluid meniscus slightly above the 0.5 ml mark, incomplete absorption of CO_2 and/or O_2, evolution of unknown gas during the analyses, or from some other cause not yet apparent. Regular setting of the fluid meniscus just above the 0.5 ml mark is possible, since one of us (F.J.K.) performed all analyses. Incomplete absorption of O_2 or evolution of unknown gas during analyses seems unlikely in view of our mass spectrometer analysis. Incomplete absorption of CO_2 remains a possibility.

The amount by which unity exceeds the ratio of V_p to V_d varies significantly with extraction chambers. The mean ratio of V_p to V_d with chamber 1 was 0.9904. The same mean ratio with chambers 2 and 3 was 0.9976 and 0.9897. When these results are used to compare chamber 2 with chamber 1 or 3, p values well under 0.01 are obtained. When chambers 1 and 3 are compared, p is between 0.2 and 0.3. This variation among extraction chambers is explainable by the fact that standard chambers are calibrated at 0.5 ml with an accuracy of ± 0.003 ml. This is ± 0.6 per cent of the 0.5 ml, and could provide as much as a 1.2 per cent difference between any two chambers. Our three mean ratios are all within a range of 0.8 per cent.

To obtain maximum accuracy with our technique then, an average correction factor must be derived for each extraction chamber. For any chamber, this factor is obviously the mean ratio of V_p to V_d. For the chambers we have used, it is 0.9904, 0.9976 and 0.9897, respectively. It should be emphasized that the use of these factors in effect standardizes this application of our technique against accepted values for N_2 and A solubility in water. These factors "compensate" for the possibilities mentioned above, as well as for slight inaccuracy in calibrating the 0.5 ml mark and any other constant factor we have overlooked.

The particular correction factors given here apply to a particular analyst's determination of a single gas mixture contained in a given liquid phase, namely 97.5 per cent N_2-2.5 per cent A in water. One may wish to apply the method reported to other gas mixtures in water, to the same gas mixture in other solvents, or to other gas mixtures in other solvents. If maximum accuracy is necessary, the individual analyst must determine other similar correction factors.

The reproducibility of our technique is assessed by examining the reproducibility of results with each of the chambers used, i.e., by examining the scatter of the ratios of V_p to V_d around the mean ratio of V_p to V_d. For chambers 1, 2 and 3, 1 standard deviation was 0.004 (0.4 per cent), 0.003 (0.3 per cent) and 0.002 (0.2 per cent), respectively. The improvement in reproducibility from the original group of analyses to the second, and from the second to the most recent, is to be noted. The standard deviation of 0.2 per cent is the maximum reproducibility achieved. A reasonable, reliable estimate of reproducibility is suggested as being the over-all standard deviation for all 46 water analyses, 0.3 per cent. This indicates that the mean corrected nonabsorbable gas content of a water sample analyzed in duplicate has a 95 per cent chance of being within 0.4 per cent of the true inert gas content (standard error = standard deviation of mean = 0.3 per cent/$\sqrt{2}$ for duplicate analyses).

SOLUBILITY OF THE INERT GAS OF AIR IN URINE AT 37° C

This method has been studied with the particular aim of determining the inert gas content of urine samples. In the process, the solubilities of a 98.8 per cent N_2-1.2 per cent A gas mixture in 50 urines at 37° C have been determined. Thirty-nine of the urines were from laboratory personnel, and 11 from patients with severe, diffuse, obstructive emphysema. A majority of samples was produced during slight or moderate water diuresis. The equilibration and transfer technique described for water was employed without modification. P_B varied between 739.5 and 752.0 mm Hg. Each urine was analyzed in duplicate with the method reported. Analysis temperatures were between 23.3 and 32.0° C. Urine osmolality, specific gravity, and chloride concentration were never altered by the equilibration process. Mass spectrometer analyses of the nonabsorbable gas being measured (Table I) showed it to be 99.5 to 99.9 per cent N_2 and A.

In applying Equation 1, the water analyses values of i and α' were again employed. Each nonabsorbable gas content was then multiplied by the water analysis correction factor for the chamber used, to give the corrected nonabsorbable or "determined inert" gas content for the analysis.

These analyses were performed by the same individual who performed the water analyses, using the same extraction chambers. The validity of using the same i, α', and chamber correction factors as in water analyses therefore depends on two assumptions: *1*) that the nonabsorbable gas being measured is 97.5 per cent N_2-2.5 per cent A, and *2*) that α' is not overestimated by a significantly different amount than that used in water analyses. Our mass spectrometer analyses support the first assumption. The second seems reasonable when one considers that a 20 per cent greater overestimation of α' in urine analysis would affect determined inert gas content by only 0.2 per cent (see Equation 1).

The inert gas solubility for each analysis was obtained from this rearrangement of Equation 2. The subscript N_2 is again omitted.

$$\alpha_{u_{37}} = \frac{V_{d_{Corr.}} \times 760}{F_I(P_B - 47.1) \times 100} \qquad [4]$$

where $V_{d_{Corr.}}$, the corrected nonabsorbable gas content, has been substituted for V_p. $\alpha_{u_{37}}$ is the Bunsen coefficient of a 98.8 per cent N_2-1.2 per cent A mixture for the urine sample at 37° C. The average difference between duplicate $\alpha_{u_{37}}$ determinations was 0.4 per cent. The average difference between duplicate water analyses was also 0.4 per cent. The mean $\alpha_{u_{37}}$ of duplicate analyses should therefore have a 95 per cent chance of being within 0.4 per cent of the true $\alpha_{u_{37}}$.

The variation of the mean $\alpha_{u_{37}}$ of duplicate analyses with osmolality, chloride concentration

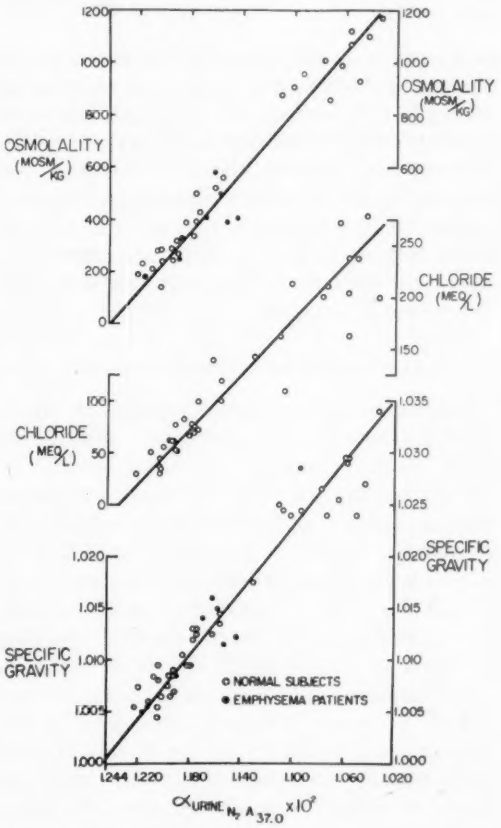

FIG. 1. THE ABSCISSA SHOWS THE SOLUBILITY OF THE INERT GAS OF AIR IN URINES OF NORMAL SUBJECTS AND EMPHYSEMATOUS PATIENTS AT 37° C. This solubility is expressed as volume gas (STP) per volume fluid per 760 mm Hg dry pressure of gas and has been plotted against osmolality, chloride concentration and specific gravity. See text for equations of regression lines and further analysis of relationships shown.

and specific gravity has been studied empirically. The results appear in Figure 1. There is no apparent difference between urines of normal subjects and those of emphysematous patients. The least squares regression lines shown have the following equations and correlation coefficients of 0.95, 0.89 and 0.94:

$$\alpha_{u_{37}} = 0.01246 - (1.76 \times 10^{-6}) \text{ (mOsm per kg)} \quad [5]$$

$$\alpha_{u_{37}} = 0.01236 - (7.57 \times 10^{-6}) \text{ (mEq Cl per L)} \quad [6]$$

$$\alpha_{u_{37}} = 0.01250 - (6.48 \times 10^{-2}) \text{ (sp gr} - 1.0000). \quad [7]$$

In Equation 7, specific gravity is expressed to the nearest 0.005 unit. An analysis of variance of each line shows that none of the abscissa intercepts (0.01246, 0.01236 or 0.01250) is significantly different from 0.01244, the solubility of a 98.8 per cent N_2-1.2 per cent A gas mixture in water. Further analysis indicates that $\alpha_{u_{37}}$ can be predicted from one or more of these parameters with an accuracy of only ± 2 per cent (and then only 95 per cent of the time).

APPLICATION OF METHOD TO BLOOD

Application of the method reported to whole blood has recently been attempted. Preliminary results seem promising. A larger amount of caprylic alcohol (10 drops) is used to minimize foaming, and a less alkaline CO_2-O_2 absorber is necessary. Twenty g of $Na_2S_2O_4$ and 2 g of sodium anthraquinone-B-sulfonate are dissolved in 100 ml of 1 N KOH and filtered rapidly through gauze. KOH, 0.5 N, does not absorb all the CO_2 in 25 ml of blood. KOH, 1.5 N or stronger, causes formation of precipitates that are extremely difficult to remove. Critical adjustment of the fluid meniscus to the 0.5 ml (or 50 ml) mark is more difficult with blood than when analyzing water or urine. Immediately after the blood sample has been introduced, the Hg in the extraction chamber is slowly raised and lowered several times. A precipitate forms as the sample and reagent are mixed. The slow mixing helps avoid this precipitate's forming in large lumps. For the same reason, the initial 1 to 2 minutes of mechanical shaking are done at low speeds.

SUMMARY

A method for determining the inert gas content of a fluid within 0.004 volumes per cent (0.4 per cent) is described. A Van Slyke manometric apparatus is used to measure the amount of gas other than CO_2 and O_2 in a 25 ml sample of the fluid. The method has been evaluated by analyzing distilled water equilibrated with air at 37° C. The solubility of the inert gas of air in various human urines has been determined at 37° C. Empirical relationships of solubility to osmolality, specific gravity, and chloride concentration are described. Preliminary results indicate that the method can be applied to blood.

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THE ARTERIAL–ALVEOLAR INERT GAS ("N_2") DIFFERENCE IN NORMAL AND EMPHYSEMATOUS SUBJECTS, AS INDICATED BY THE ANALYSIS OF URINE *

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Canfield and Rahn (2) have theorized that an arterial-alveolar difference in inert gas pressure is established whenever various units of the lung have unequal ventilation-perfusion ratios, even though the net inert gas exchange is zero.[1] The magnitude of this difference is expected to serve as an index of the variance of ventilation-perfusion ratios among the alveoli. The existence of such a difference has been shown in anesthetized dogs (2, 3). Similar measurements have not yet been made on man. The major difficulty in these determinations is the lack of a sufficiently accurate and reproducible method for measuring the partial pressure of inert gas in the arterial blood. It can be argued (see below) that urine inert gas tension reflects arterial inert gas tension. With this in mind, a method for determining the inert gas content of urine (4) has been applied to human urine in a manner believed to enable the calculation of the arterial inert gas tension within ± 2 mm Hg. Since the inert gas tension of mean alveolar air can be calculated with similar accuracy, a study of the arterial-alveolar inert gas pressure difference has been possible. This difference is +3 mm Hg in normal laboratory personnel and can be over +20 mm in patients with diffuse, obstructive emphysema.

METHODS

Seven male laboratory personnel and seven male subjects with severe, diffuse, obstructive emphysema were studied. After emptying the bladder, each subject slowly drank 0.5 to 1 L of water. Approximately one-half hour later the barometric pressure was read and the rectal temperature taken 7 to 8 cm from the external sphincter. After accumulating a sufficient amount of urine, the subject voided into the apparatus shown in Figure 1. This apparatus was designed to obtain anaerobic urine samples and was maintained at 36.5° C by a constant temperature water bath. Suspended in the bath were a long, thin separatory funnel (A) and a larger glass container (B). A three-way stopcock, which could be controlled from outside the water bath, enabled incoming urine to be directed to A or B. A was open to the atmosphere just above the surface of the water. The glans penis was pressed into a receptacle (C) just outside the water bath, and a thin, easily distensible rubber tube covering the receptacle was pulled over the distal penis to ensure a tight seal. During the first part of the voiding, urine was directed into B. After the dead space of the system had been flushed and no air bubbles remained, the stream was diverted into A. Immediately after voiding, two Ostwald-Van Slyke pipets with rubber tip and stopcock and custom-built to deliver 25 ml were inserted (in an "inverted" fashion) to the bottom of A and filled. These aliquots were used for the duplicate Van Slyke manometric analysis of the inert gas content of the bladder urine. The remaining urine was equilibrated in a tonometer with air at 37° C and also analyzed in duplicate for inert gas content, using the same Van Slyke apparatus. The detailed procedures for analysis and tonometry have been described previously (4).

A temperature slightly below body temperature (36.5° C) was chosen for the collection apparatus to avoid any change in the inert gas content of the urine between the time of voiding and the time of sampling. Since the urine could cool only slightly, its inert gas tension had to remain near that of atmospheric air. Any gas exchange that still occurred across its upper surface was not important, since pipets were filled from the lowermost part of the sample immediately after voiding. Keeping the water bath below body temperature also prevented any loss of gas because of supersaturation. It is possible that in practice a less elaborate collection apparatus may serve equally well.

Anaerobically collected urine aliquots were introduced into a Van Slyke apparatus within 5 to 30 minutes after filling of the pipets. In actual experiments, no change in inert gas content could be detected after a filled pipet had stood at room temperature for as long as 8 hours. Alcohol was prohibited for 36 hours before the experiment, since small amounts of it appear unchanged in the urine for 8 hours or more after ingestion (5). Such traces of alcohol can be measured as "inert gas" with the method of analysis

* A preliminary report of this work was presented at the April, 1959 meetings of the Federation of American Societies for Experimental Biology, Atlantic City, N. J. (1). This study was supported in part by the Air Research and Development Command, Wright-Patterson Air Force Base, Ohio.

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[1] Since the inert gas of arterial blood and alveolar air is often treated as being entirely N_2, the arterial-alveolar inert gas pressure difference has also been referred to as the arterial-alveolar "N_2" difference.

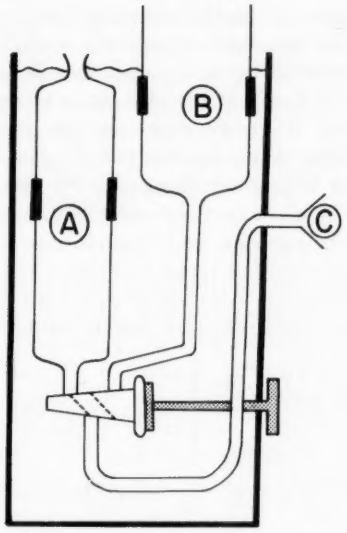

FIG. 1. APPARATUS FOR COLLECTING ANAEROBIC BLADDER URINE SAMPLES. For description, see text.

used (6). No restrictions were imposed during the period of urine formation.

Rectal temperatures were between 36.9 and 37.7° C and averaged 37.2° C. P_B ranged from 737.1 to 752.0 mm Hg. Analysis temperatures were between 23.3 and 32.0° C. Ammonia concentration of the bladder urine (7) varied from 1 to 28 mmoles per L (with one exception, which was 68 mmoles per L). Specific gravities were between 1.0045 and 1.0160, and osmolalities between 137 and 580 mOsm per kg.

CALCULATIONS

Partial pressure of inert gas in arterial blood (P_a). No method for the direct determination of inert gas tension is available. We originally hoped to calculate the inert gas tension of arterial blood by measuring its inert gas content before and after tonometry with a known inert gas tension at body temperature. Early attempts at this analysis were unsatisfactory, and we turned our attention to urine. Since one may assume no net inert gas exchange in the tissues, it might be argued that inert gas tension is the same throughout the body, i.e., $Pa_{N_2} = Pv_{N_2} = P_{tissues_{N_2}} = P_{urine_{N_2}}$. Applying considerations similar to those above, the inert gas tension of bladder urine can be obtained from this proportion:

$$\frac{P_{N_2} \text{ of bladder urine}}{P_{N_2} \text{ of tonometered urine}} = \frac{\text{inert gas content of bladder urine}}{\text{inert gas content of tonometered urine}}.$$

This is the general approach we have used. However, certain refinements, which will be discussed below, are necessitated by temperature variation within the body.

For the development of the final equation for determining the inert gas tension of arterial blood, four assumptions are necessary: *1)* The partial pressure of inert gas (N_2-A) in the urine is the same as that in the blood perfusing the urinary system. *2)* The inert gas measured in bladder urine is entirely N_2 and A, in the ratio expected after equilibration with air. *3)* The alteration in blood inert gas solubility caused by any change in blood temperature between the lung and the urinary system results in an inverse alteration of blood inert gas tension, of the same percentage magnitude. That is, $\frac{\alpha_1}{\alpha_2} = \frac{P_2}{P_1}$, where 1 and 2 represent different temperatures. *4)* Over a small temperature range, the relative change in inert gas solubility in water is the same as that in blood or urine. That is, $\frac{\Delta\alpha/\alpha}{^\circ C}$ is a constant for water, urine and blood. Near 37° C this constant is −1.2 per cent per degree C increase. (This assumption is necessitated by the lack of inert gas solubility data for blood and urine at different temperatures.)

The following symbols will be used. In order to simplify the nomenclature, the usual subscript N_2 (designating the N_2-A mixture that is the inert gas of air) will be omitted.

V_u = inert gas content (volumes per cent, STPD) of anaerobically collected bladder urine; V_e = inert gas content (volumes per cent, STPD) of urine equilibrated in a tonometer with air at 37° C; t_k = temperature (° C) at which blood and urine equilibrate within the urinary tract; t_l = temperature (° C) at which alveolar air and blood equilibrate within the lung (referred to as "lung temperature"); P_B = ambient pressure (mm Hg) during tonometry of urine; F_I = inert gas fraction of air; α_{b_k} and α_{b_l} = Bunsen coefficients of the inert gas of air for blood at t_k and t_l. Bunsen coefficients are defined as the volume of gas (STP) absorbed by 1 vol of fluid when the dry pressure of the gas is 760 mm Hg. α_{u_k} and $\alpha_{u_{37}}$ = Bunsen coefficients of the inert gas of air for urine at t_k and 37° C; p_k = partial pressure of inert gas (mm Hg) in blood perfusing the urinary system; P_a = partial

pressure of inert gas (mm Hg) in blood leaving the lungs (referred to as "arterial blood").

V_u and V_e are both the result of particular inert gas partial pressures and solubilities. If assumptions *1* and *2* are correct, V_u is given by this equation:

$$V_u = \frac{P_k}{760} \times \alpha_{u_k} \times 100. \quad [1]$$

V_e is given by this equation:

$$V_e = \frac{F_I(P_B - 47.1)}{760} \times \alpha_{u_{37}} \times 100. \quad [2]$$

Dividing V_u (Equation 1) by V_e (Equation 2) and solving for P_k, we obtain:

$$P_k = \frac{V_u}{V_e} \times F_I(P_B - 47.1) \times \frac{\alpha_{u_{37}}}{\alpha_{u_k}}. \quad [3]$$

If assumption *3* is correct, we may also say:

$$\frac{\alpha_{b_k}}{\alpha_{b_I}} = \frac{P_a}{P_k}. \quad [4]$$

Solving Equation 4 for P_a and substituting for P_k from Equation 3, we obtain:

$$P_a = \frac{V_u}{V_e} \times F_I(P_B - 47.1) \times \frac{\alpha_{u_{37}}}{\alpha_{u_k}} \times \frac{\alpha_{b_k}}{\alpha_{b_I}}. \quad [5]$$

The last two factors in Equation 5 have arisen because of slight differences among t_k, t_I and 37° C. If assumption *4* is correct, $\frac{\alpha_{u_{37}}}{\alpha_{u_k}} = 1 - 0.012(37 - t_k)$ and $\frac{\alpha_{b_k}}{\alpha_{b_I}} = 1 - 0.012(t_k - t_I)$. When these two factors are multiplied, t_k becomes insignificant and the product is $1 - 0.012(37 - t_I)$. Equation 5 therefore becomes:

$$P_a = \frac{V_u}{V_e} \times F_I(P_B - 47.1) \times [1 - 0.012(37 - t_I)]. \quad [6]$$

The similarity of Equation 6 to the proportion mentioned at the beginning of this section is to be noted. Only a single correction factor for temperature variation within the body has been added. However, this correction factor is significant.

The description of the method used for determining the inert gas content of urine (4) includes a discussion of extraction chamber correction factors for accuracy. Since P_a depends only on the ratio of V_u to V_e, these factors are not necessary if V_u and V_e are determined by the same analyst on the same extraction chamber. In other words, if one ignores the absolute values of V_u and V_e and calculates only P_a, "standardization" of extraction chambers is unnecessary. The actual pressure and temperature readings needed for the Van Slyke manometric determination of V_u and V_e by the method used (4) may now be substituted directly into Equation 6:

$$P_a = \frac{\left[\frac{P_1 - P_2}{1 + 0.00384\ t}\right]_u}{\left[\frac{P_1 - P_2}{1 + 0.00384\ t}\right]_e} \times F_I(P_B - 47.1) \times [1 - 0.012(37 - t_I)]. \quad [7]$$

Definitions of P_1, P_2 and t have been given with the description of the method. F_I has been checked on a Scholander gas analyzer (8) and was always taken to be 0.7903 (9).

Partial pressure of inert gas in mean alveolar air (P_A). P_a has been determined from urine formed over a 0.5 to 1.5 hour period. The respiratory exchange ratio may vary from moment to moment, especially in emphysematous patients. It is therefore preferable to determine the inert gas tension of mean alveolar air over the entire period of urine formation rather than at any particular instant. We therefore believe (see below) that the average alveolar inert gas tension during the period of urine formation can be calculated as accurately as it can be measured. In addition, it is difficult to determine the "ideal" method of sampling alveolar air, particularly in emphysematous patients.

Using standard respiratory physiology symbols and treating all inert gas as N_2, this equation for alveolar inert gas tension may be developed when CO_2 is absent from the inspired gas (10):

$$P_{A_{N_2}} = F_{I_{N_2}}\left[\frac{P_{A_{CO_2}}(1 - R)}{R} + (P_B - P_{H_2O})\right]. \quad [8]$$

Implicit in this equation are two assumptions: *1)* mean intra-alveolar pressure = P_B; and *2)* alveolar air is 100 per cent saturated with water

vapor. We have made two additional assumptions: *3*) R = 0.85 (in all experiments); and *4*) $P_{A_{CO_2}}$ = 40 mm Hg (in all experiments). (The reasonableness of this assumption even in the case of emphysematous patients will be pointed out below.) With these assumptions, Equation 8 may be rewritten as follows. The subscript N_2 is again omitted.

$$P_A = F_I[7.1 + (P_B - P_{H_2O})] \qquad [9]$$

where P_A = average partial pressure of inert gas (mm Hg) in mean alveolar air during period of urine formation; F_I = inert gas fraction in inspired air; P_B = ambient pressure (mm Hg) during the period of urine formation;[2] P_{H_2O} = vapor pressure of water (mm Hg) at t_1. The latter has been described previously. F_I has been checked on a Scholander gas analyzer (8) and always taken to be 0.7903 (9). Vapor pressures of water at t_1 have been obtained by interpolating values in standard tables (11).

Lung temperature. The calculations of P_a and P_A both involve t_1, the temperature at which alveolar air and blood equilibrate within the lung. We are aware of three studies that discuss the relationship of lung temperature to rectal temperature (12–14). All agree that lung temperature is slightly below rectal temperature. In the experiments reported, t_1 has always been assumed to be 0.5° C below the measured rectal temperature. This is the maximum difference observed and is least favorable from the viewpoint of demonstrating an arterial-alveolar inert gas tension difference. Rectal temperature was always measured 7 to 8 cm from the external sphincter because it gradually increases for the first 6 cm from the sphincter (15).

RESULTS

P_A and duplicate values of P_a were calculated for each experiment from Equations 9 and 7. The arterial-alveolar difference in inert gas pressure, which will be designated henceforth as aAD, was obtained by subtracting each P_A from the average P_a. Values for all experiments appear in Table I. In 18 experiments on 7 normal subjects, aAD ranged from −1.1 to +6.0 mm Hg and averaged 3.4 mm Hg. In eight experiments on seven emphysematous patients, aAD ranged from +14.3 to +29.5 mm Hg and averaged 22.2 mm Hg.

TABLE I

*The arterial-alveolar inert gas difference (aAD) in normal laboratory personnel and in patients with severe, diffuse, obstructive emphysema**

Normal subjects	aAD
	mm Hg
F.K.	0.5
F.K.	4.1
F.K.	4.3
R.K.	1.6
R.K.	5.2
R.K.	6.0
H.H.	5.2
H.H.	5.7
H.H.	0.1
J.P.	−1.1
J.P.	3.3
J.P.	−1.1
L.M.	5.0
L.M.	5.6
J.C.	5.8
J.C.	5.6
P.H.	1.7
P.H.	3.8
Emphysematous patients	
E.C.	28.0
E.S.	26.3
M.L.	20.9
P.S.	29.5
L.K.	17.7
F.Z.	15.7
A.P.	24.9
A.P.	14.3

* For calculation of aAD, see text.

DISCUSSION

Reliability of observed aAD values. To evaluate the aAD values observed, the reliability of our analytical method and the reliability of our assumptions must be considered. Our analytical method is used to determine V_u/V_e. This ratio has been determined twice in each experiment. The average difference between duplicate determinations was 0.3 per cent. For any individual experiment, it may be shown that the mean of duplicate determinations has a 95 per cent chance of being within 0.3 per cent of the true V_u/V_e. Thus, if our assumptions are correct, 95 per cent of our aAD values should be within 1.7 mm Hg (0.3 per cent)of the true aAD.

In evaluating our various assumptions, it is important to consider the errors they may cause in terms of their final influence on the aAD. The underestimation of t_1 by 0.1° C causes aAD

[2] It is to be noted that the barometric pressure has been read twice in each experiment—during the period of urine formation and during the period of urine tonometry.

TABLE II

*Mass spectrometer analyses of inert gas of bladder urine and tonometered urine**

	Specimen	N_2	A	CO_2	O_2	"Hydrocarbon"
		%	%	%	%	%
Normal subject F.K. aAD = 4.3	Bladder urine	97.7	2.0	0.05		0.25
	Tonometered urine	98.0	1.9	0.04		0.03
Emphysematous patient A.P. aAD = 14.3	Bladder urine	97.0	2.5	0.24	0.10	0.24
	Tonometered urine	97.1	2.4	0.33	0.06	0.06

* The inert gas of bladder and tonometered aliquots from single urine samples of a normal subject and an emphysematous patient were collected, as described elsewhere (4). For discussion of this table, see text. Results have been calculated on a water-free basis.

to be underestimated by 0.9 mm Hg. The overestimation of R by 0.05 causes aAD to be overestimated by 2.3 mm. The underestimation of P_{ACO_2} by 5 mm Hg causes aAD to be overestimated by only 0.7 mm. Every 1 per cent unsaturation of alveolar air with water vapor causes aAD to be overestimated by 0.4 mm. If $\frac{\Delta\alpha/\alpha}{^\circ C}$ of blood or urine differs by 50 per cent from the corresponding value for water, aAD will change by only 0.6 mm in a typical case.

The aAD values observed in emphysematous patients are even larger than might theoretically have been proposed. In attempting to account for them, we have also considered that the mean intra-alveolar pressure throughout the respiratory cycle may be greater than atmospheric in emphysematous patients. Our analyses of typical mouth-to-esophagus pressure difference tracings in quietly breathing emphysematous patients (16, 17) indicate a mean positive intra-alveolar pressure of 1 to 2 mm Hg. This would cause our emphysematous aAD values to be overestimated by approximately the same amount.

The assumption that the inert gas measured in bladder urine is entirely N_2 and A, in the ratio expected after equilibration with air, deserves particular attention. If some gas other than N_2 or A were measured in bladder urine but not in equilibrated urine, it would be incorrectly interpreted as an aAD. The evidence that this situation did not obtain in the experiments reported will now be discussed. This evidence is direct and indirect.

The direct evidence consists of the mass spectrometer analyses shown in Table II. The inert gas of bladder and tonometered aliquots from single urine samples of a normal subject and an emphysematous patient were collected, as described elsewhere (4). aAD was 4.3 and 14.3 mm Hg, respectively. The percentage of the inert gas which was N_2 and A was 99.5 to 99.9, in the ratio expected if the relative solubilities of N_2 and A are the same in urine as in water. There was never a mass 12 peak to indicate that some of the "N_2" measured was CO. The small "hydrocarbon" fraction has not been identified specifically. The trace of "O_2" (mass 32 peak) reported in the emphysematous urine may represent O_2 "carry-over" from a previous mass spectrometer analysis rather than incomplete O_2 absorption. Comparing corresponding bladder and tonometered urine analyses, there is no obvious difference between the inert gas of bladder and tonometered urine.[3] There is also no obvious difference between the inert gas of a normal subject's bladder urine and an emphysematous patient's bladder urine.

The indirect evidence lies in two areas. If ammonia occurs as part of the "inert gas" of bladder urine, aAD, as measured by our method, should increase with ammonia concentration. No relationship between the ammonia concentration of bladder urine and aAD could be found in normal subjects or in emphysematous patients. The ranges of ammonia concentration in the two groups were similar (1 to 28 mmoles per L). If some volatile substance were being excreted preferentially in the urine in significant amounts, its concentration (and therefore aAD) might be

[3] There was 0.2 per cent less "hydrocarbon" reported in each tonometered aliquot than in the corresponding bladder aliquot. If this were a true, consistent difference, it would reduce each aAD calculated by 1 mm Hg.

expected to increase with urinary solute concentration. No relationship between osmolality or specific gravity and aAD could be found in normal subjects or in emphysematous patients. The ranges of osmolality and specific gravity in the two groups were similar (137 to 580 mOsm per kg and 1.0045 to 1.0160). Unlike mass spectrometer analysis, this last evidence does not rule out the presence of some gas or other volatile substance which re-equilibrates with urinary tract capillary blood after the solute concentration of forming urine has become constant.

Evidence that gas other than N_2 and A can at times be measured in bladder urine but not in equilibrated urine is provided by the study of Van Der Aue, Brinton and Kellar (6). Bladder urine gave positive chemical tests for ethanol for 1 to 2 hours after drinking alcohol (rum). Significant increases in the differences in inert gas content between bladder and tonometered urine occurred during the same period. Such increases would account for a 10 to 15 mm Hg overestimation of aAD. When a few drops of ethanol were added to a urine just before analysis, even more striking increases in inert gas content occurred (6).

We believe that we have occasionally measured gas other than N_2 and A in bladder urine, but not in equilibrated urine in experiments performed without diuresis. The more concentrated urines obtained have had specific gravities as high as 1.0340. In eight experiments on four normal subjects, aAD ranged from -3 to $+8$ and averaged 4 mm Hg. In additional experiments on one of the same subjects (J.P.) and another normal subject (J.C.), aAD was 18 and 19 mm Hg, respectively. In one experiment on an emphysematous patient, aAD was 38 mm Hg. Specific gravities in these last three cases were 1.0270, 1.0240 and 1.0285. The inert gas contents of the tonometered aliquots were not remarkable for these solute concentrations. The cause of these occasional marked increases in aAD has not been identified. Diet preceding collection of the samples may be of interest. All samples not produced during diuresis were tested for "acetone bodies" with sodium nitroprusside-ammonium sulfate-sodium carbonate powder, and were negative. aAD values determined from diuresis urines of J.P. and J.C. have been given in Table I. The emphysematous patient was not studied during diuresis.

Interpretation of results. There is an obvious difference between the aAD values observed in normal subjects and emphysematous patients. Even the highest value in a normal subject and the lowest value in an emphysematous patient are widely separated. The aAD values observed in normal subjects suggest a small positive aAD in normal man. However, we cannot rule out the possibility of a constant error in one or more of our assumptions. aAD values nearly identical to those reported here have recently been obtained by Briscoe and Gurtner (18), using a similar experimental approach.

The possible existence of an aAD was suggested by Canfield and Rahn (2), who theorized that such a difference must be established whenever various units of the lung have unequal ventilation-perfusion ratios. The inert gas tension of alveolar gas and pulmonary capillary blood varies in such units of the lung. (This variation is a consequence of the variation in CO_2 and O_2 tension resulting from unequal ventilation-perfusion ratios.) Indeed, inert gas tension can theoretically differ by as much as 70 mm Hg in various units of the normal lung. In units of the lung with high ventilation-perfusion ratios, inert gas tension is relatively low; these units contribute more heavily to mean alveolar air than to arterial blood. In units of the lung with low ventilation-perfusion ratios, inert gas tension is relatively high; these units contribute more heavily to arterial blood than to mean alveolar air. Thus, alveolar gas and pulmonary capillary blood with different inert gas tensions contribute unequally to mean alveolar air and arterial blood, resulting in an aAD for inert gas. It is therefore suggested that the magnitude of aAD is an index of the variance of ventilation-perfusion ratios among the alveoli. The aAD values observed in normal subjects suggest that such variance exists in the healthy lung, but is small. The aAD values observed in patients indicate that a large variance exists in the emphysematous lung. Using a different experimental approach, West, Fowler, Hugh-Jones and O'Donnell (19, 20) have reached similar conclusions.

At this point it might be worthwhile to consider a brief comparison between the aAD for N_2

and the opposite gradient, the AaD for O_2. If *only* unequal ventilation-perfusion ratios exist in the lung (i.e., no diffusion barriers or shunts), then it can be shown that the O_2 difference should be equal to the sum of the N_2 difference plus CO_2 difference, provided the O_2 and CO_2 dissociation curves are linear (2). Since the O_2 dissociation curve is not linear between the venous and arterial P_{O_2}, the aAD for O_2 must always be somewhat larger than the sum of the aAD for N_2 and CO_2.

In addition we must consider the effects of diffusion barriers and shunts. These cannot, by themselves, produce a N_2 difference (2), but can have a profound effect upon the AaD for O_2. Thus an observed AaD for O_2 while breathing air provides no information as to its specific source in the pulmonary system, be it a shunt, diffusion limitation, unequal ventilation-perfusion ratio, or any combination of these factors. On the the other hand, the N_2 difference provides specific information in terms of variance of ventilation-perfusion ratio. An observed N_2 difference of 20 mm Hg, for example, indicates that the O_2 difference is likely to be considerably larger. At least 20 mm of O_2 difference must result from ventilation-perfusion ratio inequalities, while all additional differences (i.e., greater than 20 mm) are due to shunts and diffusion limitations.

Wide variance in the hematocrit of blood in the pulmonary capillaries may theoretically alter a pre-existing aAD for inert gas, but by a negligible amount. It is also of interest to consider that our emphysematous patients with arterial inert gas tension 20 to 35 mm Hg above ambient inert gas tension must consistently lose inert gas to the atmosphere through the skin. From the study of Behnke and Willmon (21), we would expect the amount of this inert gas exchange to be negligible.

In a preliminary report (1), an average aAD of 10 mm Hg was reported in normal subjects. All aAD values did not take into account the difference in temperature between the lung and rectum. When this is done, all values become similar to those reported here. This emphasizes the importance of temperature variation within the body in the calculation of the aAD.

Comparison of P_a calculated from simultaneously sampled urine and venous blood. To test further the assumption that the inert gas content of bladder urine accurately reflects P_a, we have attempted to apply our method of determining inert gas content to blood, as described elsewhere (4). This has made possible the calculation of P_a from urine and simultaneously sampled blood.

TABLE III

*Comparison of arterial inert gas tensions (P_a) calculated from simultaneously sampled urine and venous blood**

	P_a calculated from urine	P_a calculated from blood
	mm Hg	*mm Hg*
Normal subjects		
R.K. (aAD = 6.0)	566.1	563.7
P.H.	558.7	560.6
Emphysema patient		
A.P. (aAD = 14.3)	571.1	571.1

* For details of these calculations, see text.

Venous blood of two normal subjects and one emphysematous patient has been analyzed. Antecubital[4] blood, 60 to 70 ml, was drawn anaerobically in a single venipuncture near the end of the period of urine formation. The dead space of each syringe had been filled with sterile heparin. After a syringe had been filled, a small amount of Hg was introduced to facilitate thorough mixing of the blood and heparin and remixing of the blood before filling of the Ostwald-Van Slyke pipet. The latter was necessary to ensure even distribution of erythrocytes throughout the syringe, since N_2 and A are more soluble in erythrocytes than in plasma (22). The inert gas content of the blood was determined before and after tonometry with a known inert gas tension at 37° C, in the usual fashion. P_a was then calculated from Equation 7.

A comparison of the P_a values determined from urine and venous blood appears in Table III. In normal subjects, P_a calculated from venous blood was 2.4 mm Hg below and 1.9 mm Hg above P_a calculated from duplicate urine analyses. In the emphysematous patient, P_a values determined from urine and venous blood coincided within 0.1 mm Hg. aAD in this patient was 14.3 mm Hg, and, as previously de-

[4] The use of an antecubital vein assumes no significant inert gas exchange across the skin drained by the vein during the drawing of the blood sample (21).

scribed, bladder and tonometered aliquots of the same urine sample were subjected to mass spectrometer analysis.

It is interesting that Hill, Twort and Walker (23) recognized as early as 1910 that urine should reflect blood inert gas tension. In 1939, Behnke and Yarbrough (24) began to measure the inert gas content of urine as a test for bubble formation in the blood stream after exposure to increased barometric pressures. Van Der Aue and associates (6) extended this work in 1945.

SUMMARY

An arterial-alveolar difference in inert gas pressure is theoretically established whenever various units of the lung have unequal ventilation-perfusion ratios. A recent method for determining the inert gas content of a fluid has been applied to human bladder urine in a manner believed to enable the calculation of the inert gas tension of arterial blood within ±2 mm Hg (0.3 per cent). The inert gas tension of mean alveolar air can be calculated with similar accuracy and the arterial-alveolar inert gas pressure difference determined. This difference ranged from −1.1 to +6.0 mm Hg in normal subjects and averaged 3.4 mm. In patients with diffuse, obstructive emphysema, it ranged from +14.3 to +29.5 mm Hg and averaged 22.2 mm. These findings are believed to indicate a small variance of ventilation-perfusion ratios in the healthy lung and a wide variance of ventilation-perfusion ratios in the emphysematous lung.

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We are also grateful to Dr. Grosvenor Bissell, Dr. Annabel Miller and Dr. Richard Miner, Veterans Administration Hospital, Buffalo, N. Y. for allowing us to study their emphysematous patients.

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OBSERVATIONS ON THE PYROGENIC RESPONSE AND ITS APPLICATION TO THE BIOASSAY OF ENDOTOXIN

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Endotoxins prepared from gram-negative bacteria produce a wide variety of physiological reactions when administered to man and experimental animals. The intravenous injection of microgram quantities of these lipopolysaccharides into the appropriate host is followed by fever, leukopenia, thrombocytopenia, hyperglycemia, and irreversible shock. In addition, endotoxins are capable of producing the Shwartzman phenomenon, dermal necrosis, renal cortical necrosis, and hemorrhagic necrosis of certain tumors (1).

The ubiquity of endotoxins has necessitated routine screening of all fluids and medications prepared for parenteral therapy, for which the pyrogenic response of the rabbit is commonly used. However, there have been few systematic studies of the dose-pyrogenic response relationships and little is known concerning the relative susceptibilities of rabbit and man to the pyrogenic and toxic properties of endotoxin (2–5).

In this report the dose-febrile response curve for *Serratia marcescens* endotoxin is described. Data are also presented which show the relative susceptibilities of several species, including man, to the pyrogenic properties of endotoxin. An analysis of the data from an extensive series of experiments demonstrates that the febrile reaction of the rabbit provides a consistent and objective procedure for the bioassay of endotoxins.

METHOD

Endotoxin

All experiments, except where otherwise stated, were done with a single endotoxin prepared by Perrault and Shear (6) from *Serratia marcescens* (lot P-45). Serratia endotoxin, prepared in this manner, has been characterized by Rathgeb and Sylvén (7) as a phospholipid-polysaccharide-protein complex in which the nitrogen content was 1.8 per cent and the carbohydrate moiety represented 76 per cent of the total dry weight.

Animals

Rabbits. All animals were New Zealand white rabbits of both sexes, weighing 2 to 3 kg, bred at the Animal Production Unit, National Institutes of Health. Prior to pyrogen tests the rabbits were trained in wooden stocks, with loosely fitting collars as the only restraining device. Before the injection was given, 1 hour was allowed for further acclimatization to the stocks. Only animals with baseline temperatures between 38.5° and 39.9° C were used for pyrogen assay. When individual rabbits were used more than once, a 2 week period was allowed between tests to avoid pyrogen tolerance.

Cats. These were of mixed breeds and weighed 2.0 to 2.4 kg. They were kept in the laboratory for 1 week prior to use and were trained in the wooden stocks just as were the rabbits. They adapted rapidly to the restraining device and had very stable baseline temperatures.

Horses. Doses of endotoxin near the pyrogenic threshold were administered to one 500 kg female and one 300 kg male. Each horse was injected twice, with an 8 week rest period between injections.

Dogs. Beagle hounds of both sexes were obtained from local breeders. The weights ranged from 9 to 11 kg. All were docile and minimal restraint was required for injections or the recording of temperatures.

Chimpanzees. Two animals housed at the Walter Reed Army Institute of Medical Research were trained over a 2 week period. At the time of the experiment they tolerated, without restraint, temperature recording with a clinical thermometer. Both were females, weighing 12.7 and 14.5 kg, and neither had been used in other experiments for 18 months prior to the administration of endotoxin.[1]

Patients

Six patients hospitalized on the general medical service of the National Cancer Institute participated in this study. The malignancies represented among the patients included malignant melanoma, bronchogenic carcinoma, mycosis fungoides, and gastric carcinoma. None of the patients had overt evidence of liver disease, although one patient did have an elevated alkaline phosphatase and he-

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[1] The authors are indebted to Lt. Col. S. Gaines for providing these animals.

patic metastases may have been present. There were 2 females and 4 males; the weights ranged from 52 to 65 kg.

Recording of temperature

The temperatures were recorded rectally and the pyrogen tests were always conducted in a room with the same ambient temperature as that of the animal housing areas. Temperatures of rabbits, cats and horses were recorded by the use of rectal resistance coils (Telethermometer, Yellow Springs Instrument Co.), which were inserted to a depth of 4 inches or more. The rectal temperatures of the dog and chimpanzee were recorded with clinical thermometers. These two species remained free in their cages throughout the experiments. Patient temperatures were recorded at 4-minute intervals using rectal thermo-couples and an automatic multipoint recorder (Minneapolis Honeywell). Temperatures were measured immediately before injection and at 30-minute intervals thereafter until they returned to normal.

Fever index

Febrile responses were plotted on 1×1 inch standard graph paper (K. & E., 353–10) with 1° C and 1 hour equaling 1 inch. The area under the curve was measured in square centimeters with a compensating polar planimeter (K. & E., no. 4236 M). The fever index thus represents a measure of the height and duration of the febrile response (8). Certain difficulties in computing the fever index are noteworthy. The diurnal variation of normal body temperature in man was so great that temperatures seldom returned to pre-injection values. In such instances the fever curve was extrapolated to the baseline. Likewise, the body temperature of 5 to 10 per cent of the rabbits used in these experiments leveled off at 0.5 to 1.0° C above the pre-injection value. These curves, too, were extrapolated to the baseline. No difficulty was encountered in measuring the fever index for the horse and cat because the temperature of these animals always returned to pre-injection values.

Avoidance of contamination by bacterial pyrogens

Glassware, needles and syringes were baked overnight in a hot air oven at 150 to 160° C to eliminate pyrogens. Sterile, nonpyrogenic, physiological saline (Baxter Laboratories, Inc.) was used as diluent for endotoxin. It was checked periodically and was always found to be pyrogen-free.

Injections

All injections were made intravenously. Rabbits were injected into the marginal ear vein, horses into the jugular vein, cats and dogs into the cephalic vein and the chimpanzees and patients were injected into the antecubital vein. The volume of each injection was 1 ml.

EXPERIMENTAL

Dose-febrile response relationships. Measurements were made of the febrile response of the rabbit to a series of graded doses of endotoxin ranging from minimally pyrogenic to near-lethal. For the *Serr. marcescens* endotoxin this range was found to be 0.1 to 100 μg per animal. The number of animals tested for each dose was:

Dose (μg)	0.1	0.5	1	2	5	10	20	100
Number	8	6	8	4	15	15	12	6

As illustrated in Figure 1, the extremes of the dose-response curve were relatively flat; however, in the range of 1 to 20 μg per animal, the ratio of dose to febrile response was constant. A similar dose-response curve was obtained with another endotoxin, which was derived from *Salmonella typhosa* 0901 (9). It is noteworthy that the dose-response ratios for both endotoxins were constant only for doses that produced biphasic febrile responses.

Measurements of pyrogenic potency. The minimal pyrogenic dose has been frequently used to compare the pyrogenic potencies of different endotoxin preparations. However, inspection of the dose-response curve makes it clear that more precise comparisons would be expected if the amount of endotoxin were sufficient to produce febrile responses that fell on the linear portion of the curve. Dose-febrile response ratios were determined for three endotoxins; potency was calculated from these data by two methods: *1*) minimal dose required to produce 0.6° C rise (Figure 2); and *2*) minimal dose necessary to produce a fever

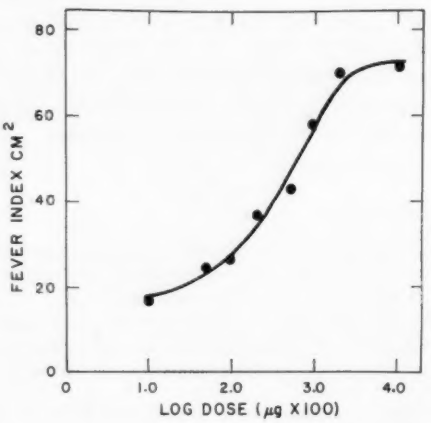

FIG. 1. LOG DOSE-FEBRILE RESPONSE CURVE OF NORMAL RABBITS TO *SERR. MARCESCENS* ENDOTOXIN. Each point represents the mean fever index of 4 to 15 animals.

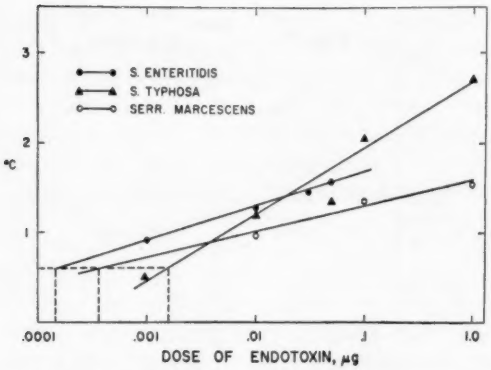

FIG. 2. DOSE-FEBRILE RESPONSE CURVES FOR THREE ENDOTOXINS. The minimal dose necessary to produce 0.6° C rise in body temperature was calculated as illustrated.

index of 40 cm², which falls on the linear portion of the dose-response curve.

It is evident from the data in Table I that the two methods of comparing pyrogenic potency yielded grossly different results. For example, when the minimal pyrogenic dose was used it appeared that *Salmonella enteritidis* endotoxin was three times as pyrogenic as *Serr. marcescens*. However, when doses necessary to produce a fever index of 40 cm² were compared, it was evident that the endotoxin from *S. enteritidis* was at least 48 times as pyrogenic as the *Serr. marcescens* endotoxin. The configuration of the dose-response curve indicates that the latter value may be a more reliable measure of over-all pyrogenic effect.

Distribution of fever indices. It is now well known that variability in the pyrogenic response of the rabbit can be significantly reduced by observing the following precautions: *1)* use of minimal restraint during the assay, preferably a loosely fitting collar; *2)* adequate training of animals (usually 1 to 2 days) so that the restraining device and temperature-recording probe are tolerated without anxiety; *3)* acclimatization to the stocks and rectal probes for 1 to 2 hours prior to injection; *4)* use of animals of uniform age and weight; and *5)* elimination of rabbits with abnormal baseline temperatures. The mean body temperature of 175 trained animals used in this laboratory was 39.2° C with a standard deviation of 0.35° C. Therefore, the temperature range of 38.5 to 39.9° C was considered normal. These findings are in satisfactory agreement with those of Lee who reported a mean temperature of 39.6° C for unrestrained normal rabbits (10).

In the course of ten experiments 85 normal rabbits that conformed to the above criteria were given a single dose of endotoxin (10 μg per animal). The frequency distribution curves for the fever indices of these animals are shown in Figure 3, which depicts the range of individual values and the arithmetic means of 85 random samples of four animals taken from this population. The mean fever index for individual animals, as well as for the random samples of four, was 52 cm². The standard deviation of the indices for individual animals was 12.8 cm²; however, the standard deviation for the means of four-animal samples was 5.9 cm². It is noteworthy that the scatter of the fever indices was about the same for any point

TABLE I

Comparison of minimal pyrogenic dose and fever index as measures of endotoxin potency

Microbial origin of endotoxin	0.6° C rise	Fever index 40 cm²
	μg/kg	μg/kg
*S. enteritidis**	0.0001	0.022
S. typhosa†	0.0012	0.028
Serr. marcescens‡	0.0003	1.052

* Supplied by Dr. Edgar Ribi.
† Supplied by Dr. Marion Webster.
‡ Supplied by Dr. Murray J. Shear.

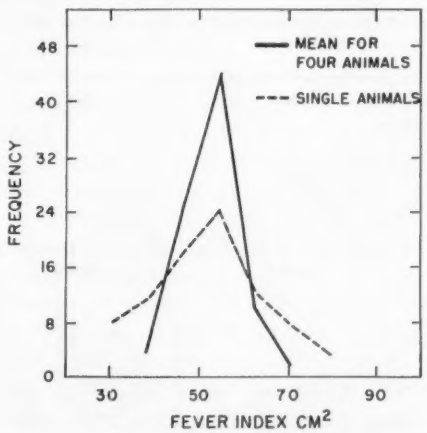

FIG. 3. COMPARISON OF THE FREQUENCY DISTRIBUTION CURVES FOR THE FEBRILE RESPONSES OF 85 INDIVIDUAL ANIMALS AND THE ARITHMETIC MEANS OF 85 RANDOM SAMPLES OF FOUR TAKEN FROM THE SAME POPULATION.

TABLE II

Febrile response of the rabbit to 20 μg of endotoxin

Fever index in cm²					
41–50	51–60	61–70	71–80	81–90	91–10
44	56	61	71	81	92
50	59	62	72	81	95
	59	63	72	82	
	59	63	72	84	
		63	73	85	
		64	75	85	
		65	75	86	
		65	76	86	
		65	76	87	
		66	77	88	
		66	77	88	
		66	79		
		68	80		
		69			
		70			
		70			

on the linear portion of the dose-response curve. The indices of 48 animals which received 20 μg of endotoxin are classified in Table II. The standard deviation of these fever indices was 11.1 cm². Similarly, the standard deviation for the febrile responses of 15 animals treated with 5 μg was 10.2 cm².

Pyrogenic response of different species to endotoxin. The febrile responses of man, cat, horse and rabbit to a near-threshold dose of endotoxin are presented in Figure 4. In each of these species, except the cat, the minimal pyrogenic dose was determined by measuring febrile responses to graded doses of endotoxin. The smallest dose that

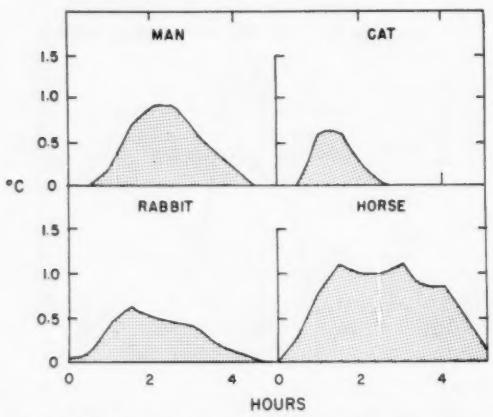

FIG. 4. THE FEBRILE RESPONSES OF FOUR SPECIES TO 0.03 μG OF ENDOTOXIN PER KG OF BODY WEIGHT.

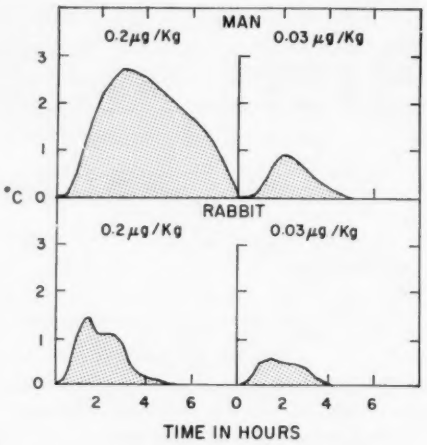

FIG. 5. COMPARISON OF THE FEBRILE RESPONSES OF MAN AND RABBIT TO DIFFERENT DOSE LEVELS OF ENDOTOXIN.

consistently produced at least a 0.5° C rise in body temperature was found to be 0.03 μg per kg for each of these species.

Although the thresholds of pyrogenic response for man and rabbit were grossly comparable, the dose-response ratio to larger amounts of endotoxin was different. Figure 5 illustrates the mean febrile responses of two male patients with bronchogenic carcinoma and malignant melanoma, and of four rabbits given 0.2 μg of endotoxin per kg. In the rabbit this dose produced a modest febrile reaction, but for the patients it was unexpectedly found to be strongly pyrogenic and very toxic. Both patients responded with profound chilling, fever, nausea, vomiting, diarrhea and hypotension. The latter persisted 36 to 48 hours and was only partially corrected by therapeutic doses of vasopressor agents and blood transfusion. Urinary output was adequate throughout the period of hypotension.

Exploratory experiments with the chimpanzee and dog demonstrated that these species were less sensitive to the *Serr. marcescens* pyrogen. Two chimpanzees receiving 0.8 μg of endotoxin per kg developed no fever. Four dogs given 1 μg per kg responded with moderate febrile reactions (mean fever index = 22 cm²).

DISCUSSION

The febrile response of individual rabbits to endotoxin, as measured by the fever index, varied

appreciably even when a number of precautions were taken to minimize variation. Nevertheless, when the febrile responses of groups of four or more animals were averaged, the means were found to be consistent and the dispersion of values was then well within acceptable limits.

The most reliable dose-related measure of the febrile response is the fever index, inasmuch as it represents an objective expression of both height and duration. Wendt, Snell, Goodale and Cranston have shown that in man the fever index is more closely correlated with the dose of endotoxin administered than is the maximum height of the fever; the mean correlation coefficients were 0.817 and 0.501, respectively. For man, however, the fever index may be a less objective measure than the maximum temperature rise because the superimposition of diurnal variations in body temperature seldom allows the post-injection temperature to return to pre-injection values (11). In contrast, the rabbit has no diurnal variations in body temperature (10), consequently the fever index is an objective measure of the febrile response of this animal. However, for the fever index accurately to represent the febrile response to a given dose, the area under the entire fever curve must be measured. Dawson and Todd considered the fever index just as variable and as poorly correlated with endotoxin dose as the maximum rise in temperature. It is noteworthy, however, that indices were determined by dropping a perpendicular from the highest temperature to the baseline and measuring the enclosed area instead of measuring the total febrile response (12).

The minimal pyrogenic dose is commonly used as a comparative measure of the pyrogenicity of endotoxin preparations. However, in the range of threshold response, doses differing by as much as fivefold to tenfold may produce febrile reactions of similar magnitude. The studies reported here indicate that more precise comparison of endotoxin preparations can be made by determining the minimum dose required to produce a mean fever index of approximately 40 cm^2 (assayed in rabbits weighing 2 to 3 kg; fever curves plotted so that 1 hour and 1° C = 1 inch). This response provides a more precise measure of pyrogenicity because it falls on the linear portion of the dose-response curve.

Knowledge of the relative sensitivity of man and the rabbit to the pyrogenic properties of endotoxin is of obvious importance, since the rabbit is generally used for the testing of biologicals prepared for parenteral therapy. Calculations from the comprehensive studies reported by Seibert (3) show that her patients manifested approximately the same susceptibility as normal rabbits to the pyrogenicity of small doses of crude bacterial filtrates. To produce comparable fevers in man and the rabbit, it was necessary to give 1.0 to 1.5 ml of bacterial filtrate per kg of body weight (3). The findings reported here provide confirmation for those of Seibert, but suggest that the dose-response curve for man is much steeper than the dose-response curve for the rabbit. It is clear that 0.2 μg of endotoxin per kg was far more toxic for patients than it was for rabbits. This finding is in agreement with those reported by Co Tui and Shrift, who administered large doses of a single pyrogenic solution to man (11 ml per kg) and rabbit (30 ml per kg) and concluded that man was at least three times as sensitive (4.1° C rise) as the rabbit (2.2° C rise) to bacterial pyrogen (2). Dare and Mogey concluded that the rabbit may be one-third to seven times as susceptible as man to threshold doses of endotoxin (4). However, those studies were carried out in both pyrogen-tolerant rabbits (one-third as susceptible) and in normal animals (seven times as susceptible). Furthermore, different criteria were employed; uncontrollable chilling was used to determine the threshold dose for man, while the minimal pyrogenic dose was used for the rabbit.

The validity of calculating the dose of endotoxin on the basis of body weight has not been clearly established. Dawson and Todd correlated the pyrogenic response of the rabbit with body weight, but the results were inconclusive (12). In the course of the studies described in this report, one 300 kg and one 500 kg horse were injected with equal total doses of endotoxin. The 300 kg horse responded with a minimal but definite fever (fever index = 12 cm^2), while the 500 kg horse developed no fever. However, in another experiment, the dose administered was calculated on the basis of body weight; this time both horses responded with comparable fever.

Endotoxins are macromolecular complexes consisting primarily of polysaccharide and firmly bound lipid with variable amounts of associated

polypeptides. These substances have been derived from a multitude of different microorganisms by a variety of different procedures. Various workers have employed endotoxin preparations which varied from crude bacterial filtrates to highly purified lipopolysaccharides, but even the purified preparations are considered to be nonhomogeneous by most physicochemical criteria. Nevertheless, all endotoxins are relatively potent pyrogens. The pathogenesis of endotoxin fever has been extensively studied, but the exact physiological mechanisms involved have not been definitely established (1). There is, nevertheless, abundant indirect experimental evidence which suggests that the febrile response is the result of a dual mechanism involving both direct stimulation of the central nervous system by endotoxin and indirect stimulation through the release of endogenous pyrogen (13, 14). It is postulated that the exogenous endotoxin damages tissues which in turn release endogenous pyrogen. The latter, along with exogenous endotoxin, stimulates the central nervous system to bring about the total febrile response (15–22). The magnitude of the fever, therefore, would depend, in part at least, upon the extent of the tissue damage and the amount of endogenous pyrogen released. Because of this and abundant other circumstantial evidence, it has been generally accepted that the pyrogenic potency of endotoxin reflects other toxic properties. Although this is a reasonable assumption, there have been no systematic studies that correlate the pyrogenic effects of endotoxin with other toxic host responses. Recent studies in this laboratory have shown that the degradation of endotoxin by plasma enzymes results in the loss of pyrogenicity as well as other toxic properties. Therefore, on the basis of the available information it seems reasonable to conclude that the pyrogenic reaction to endotoxin does, indeed, reflect general toxicity as measured by other host responses.

SUMMARY

The febrile response of the rabbit has been studied with respect to its use as an assay for endotoxin. Dose-response relationships have been established for a single endotoxin. The mean pyrogenic response of four or more animals, as measured by the fever index, was shown to be highly consistent and to afford a wide range of dose-related measurements. A standard method for comparing the pyrogenic potency of endotoxin preparations is suggested.

Man, cat, horse and the rabbit were found to have the same approximate threshold to pyrogenic stimulation by endotoxin. However, larger doses may be more pyrogenic and more toxic for man than for the rabbit. Both dog and chimpanzee were notably less susceptible to the pyrogenicity of endotoxin than the other species studied.

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INACTIVATION OF ENDOTOXIN BY A HUMORAL COMPONENT. VII. ENZYMATIC DEGRADATION OF ENDOTOXIN BY BLOOD PLASMA *

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The pathogenesis of the febrile response that follows the intravenous injection of bacterial endotoxin has been the subject of extensive investigations. In the course of these studies it became evident that blood serum contains factors which, when interacted *in vitro* with endotoxin, may either enhance or inactivate the fever-producing effects. LeQuire demonstrated that a brief period of interaction between serum and endotoxin results in augmentation of pyrogenicity (3). Others have confirmed this observation and have, in addition, defined some of the conditions necessary for demonstrating the pyrogen-enhancing effect of serum (4–8).

In seeking to explain the termination of endotoxin fever, Hegemann postulated that this was effected by components of serum capable of detoxifying endotoxin. He demonstrated the presence of such agents in serum by incubating endotoxin and serum for prolonged periods at physiological temperature (9–12). However, these experiments did not provide evidence that this effect of serum was operative in the termination of endotoxin fever.

The observations of LeQuire and of Hegemann were confirmed by Cluff and Bennett (8), who clearly distinguished the two effects of serum by demonstrating that pyrogen augmentation was not dependent upon the time or temperature of incubation and that this effect of serum was due to a relatively heat-stable factor. Inactivation of pyrogen, on the other hand, was dependent upon a heat-labile factor and was evident only after prolonged incubation at physiological temperature (8).

Other investigators working on different facets of the problem have made it evident that reaction of serum and endotoxin under appropriate conditions results in the inactivation of every host-reactive property of endotoxin thus far examined (13–20) as well as in striking alterations in the immunochemical properties of the lipopolysaccharide complex (7, 19).

However, the mechanism by which endotoxin is inactivated by plasma has remained uncertain. Studies reported here show that inactivation of endotoxin by plasma *in vitro* has the characteristics of an enzyme-catalyzed reaction.

MATERIALS AND METHODS

The experiments were carried out with endotoxin derived from *Serratia marcescens* (lot P-45). The chemical composition and physical properties of this preparation were summarized in the preceding paper (21). Pyrogen assays were carried out in rabbits under experimental conditions that conformed to the specifications described previously. All injections were made intravenously. The methods used in computing the fever index, recording of temperatures, and avoiding pyrogen contamination of glassware, needles, and syringes are described in the preceding report (21).

Plasma. Because calcium was found to inhibit partially the inactivation of endotoxin by serum (22), either citrated or resin-treated (Fenwal ion-exchange pack no. JB-2) plasma was used. Human blood was collected through an ion-exchange resin to prevent clotting. Blood from experimental animals was collected under aseptic conditions in sodium citrate (0.02 M); plasma from three or more animals was pooled in each instance, and was stored at − 20° C. All samples were demonstrated to be nonpyrogenic in doses of 1 ml per animal (the largest dose administered).

* In previous reports it was convenient to use a brief, tentative designation (EDC) for the component in blood responsible for the detoxification of endotoxin *in vitro*. More recent studies in this Laboratory indicate that there may be more than one host enzyme capable of catalyzing this reaction; e.g., endotoxin can be inactivated *in vitro* by tissue homogenates under conditions which differ from those required for detoxification by blood (1, 2). In the present report, the host product employed as a source of endotoxin-inactivating enzyme is referred to, simply, as plasma or serum.

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Reaction mixtures. Endotoxin in 0.2 to 4.0 ml of normal saline was added to the appropriate dilution of plasma. The concentrations of endotoxin and of plasma in the reaction mixtures were expressed as micrograms per milliliter and per 100 ml, respectively. The pH was adjusted by the addition of concentrated hydrochloric acid or sodium hydroxide. Pyrogen-free Ringer's phosphate buffer containing 0.132 M NaCl, 0.005 M KCl, 0.001 M KH_2PO_4, and 0.01 M NaH_2PO_4 was used as diluent.

Conditions of reaction. As shown in the preceding paper, dose-related pyrogen assays yield the highest precision when performed in the linear portion of the dose-febrile response curve (21). Thus, in the studies reported here the conditions for each experiment were selected so that the febrile responses fell on that portion of the dose-response curve. The specific conditions employed in the reaction of plasma with endotoxin are described for each set of experiments.

Calculation of reaction rate. The degradation of endotoxin by plasma was followed by measuring the pyrogenicity of an aliquot of the reaction mixture before and after incubation. The pyrogenic response to the unincubated aliquot served as a control for determining the amount of endotoxin degraded in a given time interval. In the assays, groups, of four animals each, were given equal aliquots of the unincubated or incubated reaction mixture. The mean fever index for each group provided a relative measure of endotoxin concentration. The rate of degradation was calculated according to the following equation: Rate = (A–B)/T, where A represents the mean fever index following the injection of an aliquot of the unincubated mixture; B, the mean fever index following the injection of an aliquot of incubated mixture; and T, the duration of incubation in minutes. The rate of degradation is thus expressed as centimeters^2 per minute.

Induction of endotoxin tolerance. On Days 1 and 2, animals were given 10 μg; on Day 3, 20 μg; and on Days 4 through 8, they received 50 μg daily. Tolerance to a dose of 10 μg was demonstrated on the ninth day, after which the animals were again given 50 μg of endotoxin. Thus, on the day before the crucial experiment, which was carried out on the tenth day, the animals received a total of 60 μg of endotoxin.

Tumor-bearing mice. CAF$_1$ mice of both sexes weighing 19 to 22 g were obtained from the Animal Production Unit, National Institutes of Health. Sarcoma 37 was implanted into the thigh muscle, as previously described (23), and the induction of hemorrhagic necrosis by endotoxin was determined by necropsy 18 to 24 hours after the test injection which was given on the sixth day of tumor growth. The response was graded from 0 to 4+ depending upon the extent of induced tumor necrosis. Only severe, unequivocal, hemorrhagic necrosis scored as 3+ and 4+ was considered a positive reaction.

Adrenalectomized mice. CAF$_1$ male mice were adrenalectomized under ether anesthesia 24 hours before injection. Thereafter, the animals were maintained on normal saline and food *ad lib.* Lethality was recorded 48 hours after the test injection of endotoxin.

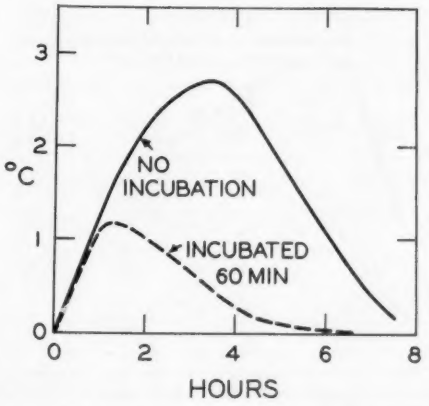

FIG. 1. THE FEBRILE RESPONSE TO EQUAL ALIQUOTS OF A PLASMA-ENDOTOXIN REACTION MIXTURE BEFORE AND AFTER INCUBATION. *Conditions of reaction:* resin-treated human plasma 80 per cent, concentration of endotoxin 200 μg per ml, pH 7.4, incubated 60 minutes at 37° C. *Aliquot tested:* 0.10 ml per animal.

EXPERIMENTAL

As will be shown, many variables were capable of affecting the capacity of plasma to degrade endotoxin. However, under appropriate conditions large amounts of endotoxin were rapidly degraded. Figure 1 illustrates the mean febrile response of two groups of four animals each. One group was given an aliquot of unincubated endotoxin and plasma; the second group received an aliquot of the same reaction mixture after 1 hour of incubation at 37° C. The mean fever indices were 78 and 20 cm^2, respectively.

Effect of plasma on the lethal, tumor-necrotizing, and pyrogenic properties of endotoxin

The data in Table I, obtained with a single reaction mixture in three different kinds of bioassay, serve to emphasize that reduction of the pyrogenic potency of endotoxin was consistently accompanied by reduction of other toxic properties.

Other experiments showed that large doses of plasma-degraded endotoxin failed to produce leukopenia when injected into normal rabbits and were not lethal for animals with reticuloendothelial blockade.

Heat lability

Heating of plasma at 50° C resulted in a progressive diminution of enzymatic activity (Table

TABLE I

Inactivation by plasma of three biological properties of endotoxin *

Endotoxin	Incubation	Mean fever index	Tumor necrosis†	Lethality‡
μg/animal		*cm²*		
2	−	39	10/10	9/10
	+	11	1/10	0/10
4	−	55	10/10	10/10
	+	11	2/10	0/10
8	−	78		
	+	25		

* Reaction conditions: resin-treated human plasma 80%, endotoxin concentration 200 μg per ml, pH 7.2, incubated 60 min at 37° C.
† No. of necrotic tumors per no. tested.
‡ Adrenalectomized mice: no. dead per no. tested.

II). In these experiments the concentration of plasma in the reaction mixtures was limited to 36 per cent, so that small changes in enzymatic activity would be more readily discernible. However, when the plasma concentration was doubled it was evident that, under these conditions of heating, destruction of enzyme was only partial.

Factors which alter the rate of endotoxin degradation by plasma

A. Temperature of incubation. Below the physiological temperature, degradation of endotoxin by plasma proceeded slowly (Figure 2). The rate was doubled on increasing the temperature of incubation from 40° to 50° C. To be sure that substrate (endotoxin) and enzyme would remain in excess throughout the period of incubation, the reaction time was limited to 10 minutes for the

TABLE II

Heat lability *

Pretreatment of plasma	Incubation at 37° C	Mean fever index†
	min	*cm²*
None	None	68
None	60	44
60 min at 37° C	60	43
15 min at 50° C	60	47
30 min at 50° C	60	54
60 min at 50° C	60	72

* Reaction conditions: resin-treated human plasma 36%, endotoxin concentration 142 μg per ml, pH 7.1, incubated 60 min at 37° C.
† Aliquot tested: 0.14 ml per animal.

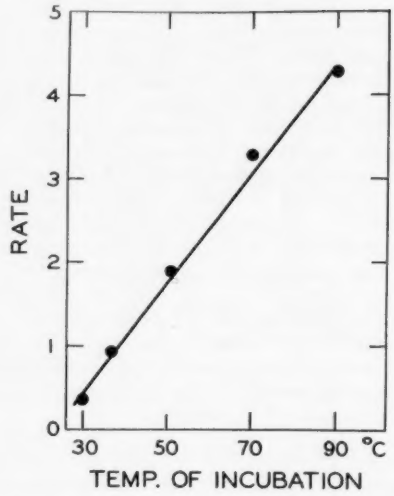

FIG. 2. THE RATE OF ENDOTOXIN INACTIVATION BY PLASMA AT VARIOUS TEMPERATURES OF INCUBATION. *Conditions of reaction:* resin-treated human plasma 72 per cent, concentration of endotoxin 142 μg per ml, pH 7.3, incubated 10 to 30 minutes. *Aliquot tested:* 0.14 ml per animal.

higher temperatures (70° and 90° C), and to 30 minutes for the lower temperatures. These findings indicate that at higher temperatures the degradation of endotoxin occurred more rapidly than heat destruction of the enzyme.

B. Concentration of endotoxin in the reaction mixture. The enzymatic nature of the reaction between plasma and endotoxin was fully appreciated only after it was observed that the degradation of endotoxin proceeded more rapidly in reaction mixtures containing high concentrations of endotoxin than in mixtures containing low concentrations. Experiments were then designed to study the influence of endotoxin concentration on the reaction velocity. Reaction mixtures containing endotoxin in concentrations ranging from 7.5 to 194 μg per ml were incubated with an excess of plasma. The biological assay was not sufficiently sensitive to determine initial reaction velocity; accordingly, the assays were performed with a reaction time of 30 minutes. When the velocity of the reaction was plotted against concentration of endotoxin (Figure 3), a mixed order reaction curve was obtained. Below 80 μg per ml the rate of endotoxin degradation was dependent upon the concentration of endotoxin. However, above 100

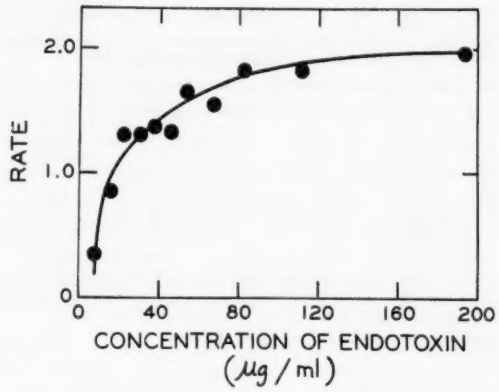

FIG. 3. THE EFFECT OF CONCENTRATION OF ENDOTOXIN ON THE RATE OF INACTIVATION BY PLASMA. *Conditions of reaction:* citrated rabbit plasma 70 per cent, pH 7.6, incubated 30 minutes at 37° C. *Aliquot tested:* 0.10 to 2.67 ml per animal.

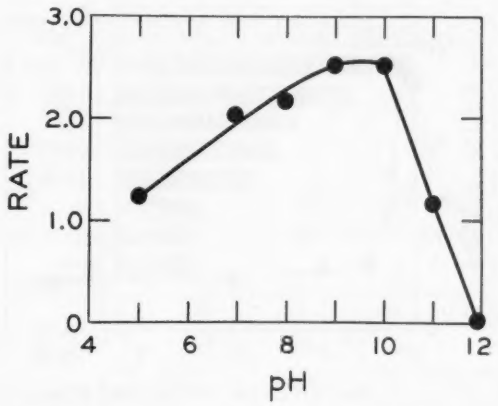

FIG. 5. THE RATE OF ENDOTOXIN INACTIVATION BY PLASMA IN REACTION MIXTURES OF DIFFERENT PH. *Conditions of reaction:* citrated rabbit plasma 36 per cent, concentration of endotoxin 142 μg per ml, incubated for 15 minutes at 37° C. *Aliquot tested:* 0.14 ml per animal.

μg per ml the rate was not significantly influenced by increased concentration. Under the specific conditions of this experiment, the Michaelis-Menten constant was estimated to be 17 μg per ml.

C. Concentration of plasma in the reaction mixture. Reaction mixtures containing an excess of endotoxin (142 μg per ml) and concentrations of plasma ranging from 18 to 72 per cent, demonstrated that the velocity of degradation is also dependent upon the concentration of plasma in the reaction mixture. Figure 4 shows the reaction rates for different plasma concentrations. The reaction time was limited to 30 minutes so that the concentration of substrate would remain in excess throughout the period of incubation. The linear relationship between reaction velocity and plasma concentration indicated that this was accomplished and that the reaction rate in this situation was altered only by variation in plasma concentration.

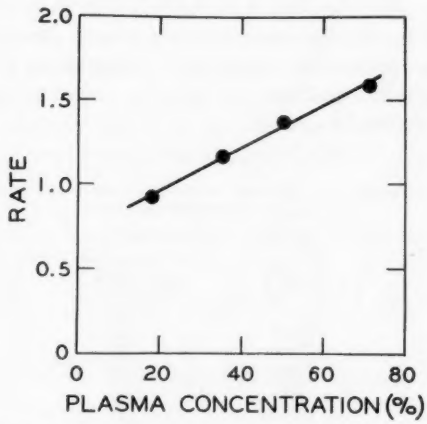

FIG. 4. EFFECT OF THE CONCENTRATION OF PLASMA ON THE RATE OF ENDOTOXIN INACTIVATION. *Conditions of reaction:* citrated rabbit plasma 18 to 72 per cent, concentration of endotoxin 142 μg per ml, pH 7.6, incubated 30 minutes at 37° C. *Aliquot tested:* 0.14 ml per animal.

D. pH of the reaction mixture. The degradation of endotoxin by plasma proceeded over a wide pH range, but the optimum was between 9 and 10 (Figure 5). In this experiment the reaction time was limited to 15 minutes so that the febrile responses to endotoxin, reacted with plasma at pH 9 to 10, would fall in the linear portion of the dose-response curve. It was shown in control experiments that the pH of comparable reaction mixtures did not change significantly during this period of incubation.

Pyrogenic properties of reaction products

Early in the course of these studies the question arose as to whether the pyrogenic activity, remaining after prolonged incubation of endotoxin with plasma, is due to products of the reaction or to unaltered residual endotoxin. The data illustrated in Figure 6 were obtained by sampling a single reaction mixture periodically during 24 hours of incubation. At physiological temperature and pH,

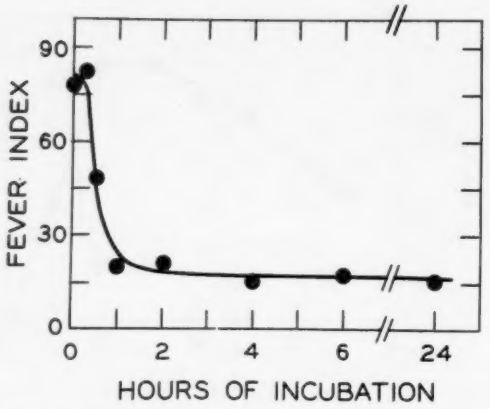

FIG. 6. THE PROGRESSION OF ENDOTOXIN INACTIVATION BY PLASMA DURING PROLONGED INCUBATION. A single reaction mixture was periodically tested for pyrogenicity during 24 hours of incubation. *Conditions of reaction:* resin-treated human plasma 80 per cent, concentration of endotoxin 200 μg per ml, pH 7.4, incubated 24 hours at 37° C. *Aliquot tested:* 0.10 ml per animal.

and after 1 to 2 hours of incubation, the febrile responses to aliquots of the reaction mixture remained constant.

There are several factors to be considered as possibly accounting for the apparent lack of further degradation of endotoxin after 1 to 2 hours of incubation. Gradual deterioration of the enzyme, because of its heat-lability, was ruled out since heating of plasma for 1 hour at 37° C did not alter the level of enzymatic activity (Table II). Inhibition by reaction products appears to be a relatively minor factor in view of the great excess of enzyme. The possibility that the reaction products are pyrogenic is considered in a later section. However, the concentration of residual endotoxin (substrate) in the reaction mixture may well be the limiting factor. After 2 hours of incubation the data indicate that approximately 99 per cent of the endotoxin originally present had been degraded, i.e., the concentration of endotoxin would be about 2 μg per ml.

Table III shows that rabbits made tolerant to endotoxin were also tolerant to the residual pyrogen in the incubated reaction mixture. Each of six normal rabbits was given 10 μg aliquots of a reaction mixture which had been incubated for 1 hour. Subsequently, the same animals were made tolerant to endotoxin by daily injections of large

TABLE III

Residual pyrogenicity of plasma-endotoxin reaction mixtures in normal and tolerant animals *

Recipients	Fever indices of individual animals						Mean fever index†
	A	B	C	D	E	F	
							cm²
Normal	20	26	29	21	18	34	25
Tolerant	1	3	2	10	4	7	4

* Reaction conditions: citrated rabbit plasma 98%, endotoxin concentration 10 μg per ml, pH 7.6, incubated 60 min at 37° C.
† Aliquot tested: 1 ml per animal.

doses of endotoxin in physiological saline. After nine daily injections the animals were again challenged with 10 μg aliquots of the plasma-endotoxin reaction mixture. These findings provide additional evidence that the residual pyrogen is undegraded endotoxin.

Experiments were next performed to obtain more direct information on the capacity of the reaction products to produce fever. Five separate reaction mixtures in which the endotoxin concentration was varied between 72 and 500 μg per ml were incubated at 37° C for periods ranging from 2.5 to 5 hours; the pH was varied from 7.4 to 9.6 (Table IV). These various reaction mixtures were tested for pyrogenic activity in rabbits. Although the dose of degraded endotoxin per animal was varied 50-fold, there was no significant difference in the mean fever index. Since the amount of reaction products also varied 50-fold, these findings indicate that the residual pyrogenicity is due not to the reaction products, but rather to undegraded endotoxin.

TABLE IV

Pyrogenicity of reaction mixtures containing different quantities of degraded endotoxin *

Dose per animal†	Mean fever index‡
μg	*cm²*
10	23
20	32
40	28
100	27
500	29

* Reaction conditions: resin-treated human plasma 72%, endotoxin concentration 72 to 500 μg per ml, pH 7.4 to 9.6, incubated 2.5 to 5 hrs at 37° C.
† In terms of content of endotoxin before incubation.
‡ Aliquot tested: 0.14 to 1.0 ml per animal.

Inhibition by divalent cation

In studies previously reported from this laboratory it was shown that calcium, barium, magnesium and manganese divalent cations inhibit the enzymatic degradation of endotoxin by plasma (*22*). With the pyrogen assay, the previous observations on the inhibitory effect of divalent cation were extended to still other cations. The experiments were carried out with a limiting concentration of resin-treated plasma so that even relatively small inhibitory effects would be detected. To prevent clotting, resin-treated plasma was heparinized (0.2 mg per ml) prior to the addition of divalent cation, all of which were tested in a concentration of 1×10^{-3} M. In control tests without plasma these cations, incubated with endotoxin alone for 30 minutes, produced no alteration in pyrogenic potency. For calculation of the relative inhibitory effects of these cations, the pyrogenic response to an unincubated plasma-endotoxin mixture was assigned the value of 100 per cent while the response to the incubated mixture was designated zero. All six cations inhibited the degradation of endotoxin by plasma; however, calcium was more inhibitory than the others (Table V). Differences in the test conditions presumably account for the differences in the effect of cuprous ion from that obtained in some earlier experiments (*22*).

Comparison of plasma from different species

Although citrated plasma from rabbit and man were about equally effective in degrading endotoxin, wide variations were found in this ability of plasma from other species. Similar reaction mixtures were prepared with pooled citrated plasma from eight species. The concentration of plasma was limited to 36 per cent, while the concentration of endotoxin was in excess. After 1 hour of incubation the residual pyrogenicity was measured in assays of equal aliquots from each reaction mixture. In control tests, aliquots of each of the plasmas were found to be pyrogen-free (fever indices of 4 cm^2 or less), except those of the cat and dog, which produced febrile responses of 9 and 10 cm^2, respectively. Figure 7 illustrates the relative effectiveness of plasma from eight species to degrade endotoxin when reacted under the same conditions. Guinea pig plasma was weakest in this reaction; however, it was not devoid of enzymatic activity. Separate experiments, in which reaction mixtures were prepared at pH 9.5 and the concentration of guinea pig plasma was increased to 72 per cent, demonstrated that the fever index could be reduced to 47 cm^2 after 1 hour of incubation. Nevertheless, even under these favorable conditions, the degradation proceeded far more slowly than with plasma from other species.

TABLE V

Inhibition by divalent cation *

Divalent cation	Inhibition†
1×10^{-3} *M*	%
Ca	68
Cu	48
Hg	42
Mg	41
Zn	39
Mn	30

* Reaction conditions: resin-treated human plasma 32%, endotoxin concentration 142 μg per ml, pH 7.2, incubated 30 min at 37° C.

† Complete inhibition = 100%.

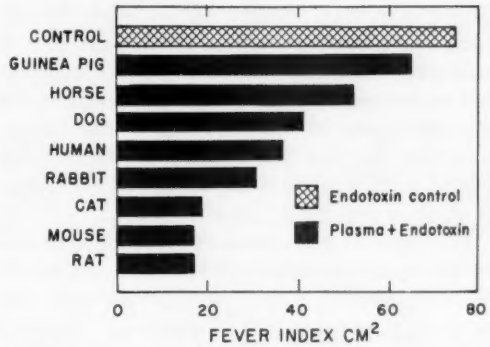

FIG. 7. THE CAPACITY OF PLASMA FROM DIFFERENT SPECIES TO INACTIVATE ENDOTOXIN *IN VITRO*. *Conditions of reaction:* citrated plasma 36 per cent, concentration of endotoxin 142 μg per ml, pH 7.1 to 7.4, incubated 60 minutes at 37° C. *Aliquot tested:* 0.14 ml per animal.

DISCUSSION

It is now clear that serum normally possesses components which may either augment or reduce the pyrogenicity of endotoxin, depending upon the conditions of reaction. This investigation showed that reduction of pyrogenic potency is dependent upon pH and adequate concentration of plasma and of endotoxin, as well as upon the time and temperature of incubation. In addition, minute

quantities of many divalent cations, especially calcium, nonspecifically inhibit the degradation of endotoxin by plasma. Rosen, Skarnes, Landy and Shear, working in this laboratory, demonstrated that differences in concentrations of calcium account for the fact that citrated or resin-treated plasma is more active in degrading endotoxin than is serum or heparinized plasma (22).

Investigators who demonstrated augmentation of the pyrogenic effects of endotoxin by serum generally employed reaction mixtures that contained low concentrations of endotoxin. Furthermore, augmentation was evident after incubation of serum with endotoxin at refrigeration temperatures, or even at 37° C, provided the time was sufficiently short (3, 4, 6, 8). The data in this report show that these conditions of reaction are unfavorable for the degradation of endotoxin. Therefore, the augmenting effects of serum would be expected to overshadow the pyrogen-inactivating effects.

On the other hand, investigators who employed reaction mixtures which contained high concentrations of endotoxin, or mixtures that were incubated for prolonged periods at 37° C, usually observed a reduction in the pyrogenic and other toxic properties of endotoxin (8, 10, 14–18). In much of the earlier work, however, insufficient attention was given to the critical importance of the relative concentrations of the reactants. For example, the concentration of endotoxin was varied widely, primarily to conform to the range of measurement and the sensitivity of the particular assay used. Now that the enzymatic nature of the reaction is known, more definitive data can be obtained if reaction mixtures are prepared with primary consideration upon the concentrations rather than on the amount of endotoxin required for a particular assay procedure.

Although it is now evident that the inactivation of endotoxin by plasma is an enzyme-catalyzed reaction, it remains for future work to identify the reaction products. In the series of experiments described here, the progress of the reaction was followed by measuring residual substrate (endotoxin) concentration. Because the fever index was consistent over a wide range of dose-related measurements it was particularly useful for this purpose. Employment of this assay as a measure of residual endotoxin is based on the inference that the residual fever-producing substance in plasma-endotoxin reaction mixtures is unaltered endotoxin.

Grant and Whalen have suggested that so-called endogenous pyrogen, which is present in serum of febrile animals 2 hours after the injection of endotoxin, is exogenous endotoxin which has been altered by host plasma (4). This suggestion prompted experiments, some of which are described here, to determine whether the residual pyrogen of incubated plasma-endotoxin reaction mixtures had the pyrogenic properties of endogenous serum pyrogen. It was readily shown that animals tolerant to endotoxin were also tolerant to the residual pyrogen. In addition, daily injections of the residual pyrogen induced endotoxin tolerance. Perhaps the most distinctive quality of endogenous pyrogen is that it is equally pyrogenic for normal and endotoxin-tolerant animals. Therefore, it would appear that the residual pyrogen of incubated plasma endotoxin reaction mixtures is not endogenous pyrogen.

The differences in the enzymatic activity of citrated plasma collected from different species remain unexplained. It was suspected that these differences might be more apparent than real and that they could be due to variations in levels of the pyrogen-augmenting factor. An exploratory experiment showed that plasma from rat (most active) and guinea pig (least active) were equally capable of augmenting the pyrogenic effects of endotoxin. However, further study is needed before this possibility can be eliminated. All plasma samples were citrated to the same extent, but there were necessary variables in the techniques used to collect the blood. For example, some of the animals were anesthetized and some were not. Also, some species were exsanguinated, whereas relatively small samples of blood were collected from others. However, in this respect it is noteworthy that identical techniques were used in collecting blood from guinea pig (least active) and rat (most active).

Although the susceptibility of different species to endotoxin varies widely, all possess, to a greater or lesser degree, the capacity to detoxify these noxious agents. The enzymatic properties of plasma described here might conceivably be implicated in the defense of the host against endotoxin. However, it is noteworthy that lethal doses of

tissue-destructive agents such as ionizing irradiation (24) and nitrogen mustard markedly reduce host resistance to infection, but leave the level of endotoxin-degrading enzymes apparently unaltered (25). Furthermore, it was shown that the plasma level of such enzymes in different species bore no discernible relationship to their susceptibility to endotoxin. Indeed, they may simply represent tissue enzymes which have been shed into the circulation, either for excretion or transport to other cells (26, 27); nevertheless, the present findings do not exclude the possible participation of this plasma enzyme in host resistance.

It has been repeatedly shown that cells of the reticuloendothelial system clear endotoxin from the circulation and that damage to these cells renders the host more susceptible to the toxic effects of endotoxin (28). Recent experiments in this laboratory have shown that soluble endotoxin-degrading enzymes are extractable from many tissues, including kidney, liver and leukocytes (1, 2). Consequently, it is possible that the enzyme in plasma originates from a variety of tissues, including the reticuloendothelial system; the presence of such enzymes in phagocytic cells may prove to be of special significance in host resistance to the noxious effects of endotoxin.

SUMMARY

The inactivation of endotoxin by blood plasma was found to have the characteristics of an enzyme-catalyzed reaction. The reaction was dependent upon the concentration of reactants and the pH of the reaction mixture, as well as on the time and temperature of incubation. Products of the enzymatic degradation of endotoxin by plasma were nonpyrogenic.

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DIRECT RENAL ACTION OF SOME DIGITALIS STEROIDS *

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Some digitalis steroids alter the activity of cardiac muscle. Applying the bioassay technique in cats and frogs, Chen and co-workers (1–4) screened many structural variants of the clinically useful digitalis compounds for cardiac action. They were able to distinguish the active from the inactive steroids by their chemical structure. The major features common to those compounds exhibiting cardiac activity include a cyclopentanophenanthrene nucleus with a hydroxyl substituent at the C-14 position and an unsaturated, 5 or 6 membered lactone ring in β orientation at C-17. Those compounds in which the lactone ring was either saturated or in α orientation lacked cardiac activity. Synthesis of active digitalis steroids without the C-14 hydroxyl has not been accomplished to date and, therefore, the role of this constituent is unknown. In addition to their familiar actions on cardiac muscle, these steroids affect cation movement in several tissues (5–15). Inhibition of the active ion transport mechanism is the common effect produced. Glynn (9), Schatzmann (5), and others (6–8), for example, have shown that the cardiac active steroids, in a concentration of about 1 μg/ml, depress potassium influx and sodium efflux in the human red cell. Depending upon the cell type, passive diffusion fluxes may or may not be inhibited. Glynn also studied certain molecular requirements for this inhibitory effect and found them identical to those necessary for cardiac activity. He concluded that the configuration at C-17 of the steroid nucleus is important and that saturation of the lactone ring greatly reduces activity.

The first data suggesting that digitalis steroids might directly affect renal electrolyte transport were reported by Farber, Alexander, Pellegrino and Earle (16). In their experiments, digoxin was administered intravenously to human subjects in the course of cardiac and renal hemodynamic studies. In several instances an increase in water and electrolyte excretion preceded any alteration in glomerular filtration rate or renal plasma flow.

With this suggestive observation, Hyman, Jaques and Hyman (17) injected digoxin directly into one renal artery of a dog and observed an increase in water and sodium ion excretion from the injected kidney. More recently, Orloff and Burg (18) observed a unilateral diuresis subsequent to the injection of strophanthidin into the leg vein of the chicken. Administered by this route, the aglycone bypasses the glomeruli and is delivered to the renal parenchyma directly via the peritubular capillaries.

Schatzmann, Windhager and Solomon (19) concluded that ouabain inhibits sodium ion reabsorbtion in the single perfused proximal tubule of Necturus extending the evidence for its direct renal action.

Our work was undertaken to answer the following questions: *1*) Are the compounds that are active in the heart and in red cell suspensions also active in the kidney? In other words, do the same structural requirements for activity in these other tissues also apply to the kidney? *2*) What are the alterations in renal hemodynamics and urinary electrolyte excretion produced by these substances?

To these ends, we injected nine structural variants of the cardiac steroids into one renal artery of dogs. By analyzing urine samples collected separately from each kidney our experiments had the virtue of being simultaneously controlled, since, with the exception of the renal arterial infusate, the two organs had identical environments. When administered intra-arterially, only those compounds known to have activity either on the heart and/or on red cell ion movement depress sodium reabsorbtion in the

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kidney. This inhibition might occur throughout the nephron or at some more localized site. Partial inhibition of the proximal transport mechanism is suggested by sodium excretions which at times exceeded 35 per cent of the filtered load.

METHODS

Dogs were anesthetized with pentobarbital. A load of 800 to 900 ml of saline was infused intravenously in 1 hour. Exogenous creatinine and para-aminohippuric acid (PAH) clearances were measured to approximate glomerular filtration rate and renal plasma flow, respectively. Appropriate amounts of these materials were dissolved in a saline infusion that was administered at a rate of 5 ml per minute for the duration of the experiment.

Through a supra-pubic incision, the ureters were catheterized at the point of their junction with the bladder. The renal artery was approached through a retroperitoneal flank incision. A curved no. 25 needle fixed to a plastic catheter was introduced into the lumen of the vessel permitting infusion of the renal artery at a rate of 12 to 24 ml per hour. Normal saline was infused into the artery during the control periods. The steroids were dissolved in 3 to 5 ml of ethanol with 10 to 20 ml of saline.

The experiments described in Table I serve as controls for the effect of this infusion on renal functions. Studies were begun when urine flow had stabilized and was approximately equal from the two kidneys. Experiments were discarded when creatinine clearances of the two kidneys differed more than 10 per cent from each other.

Conventional clearance techniques were employed with separate collections of urine from the two kidneys. Creatinine was analyzed by the Phillips modification of the Jaffé reaction (20), and PAH, by the method of Smith and associates (21). Sodium and potassium were determined by flame photometry utilizing lithium for internal standardization.

RESULTS

A total of 29 experiments is included in this report. A minimum of two experiments was performed with each of the nine compounds under study.[1] Compounds classified as "diuretic" are those that increased urine flow and sodium excretion on the injected side within 3 hours of administration. Using this restrictive definition there was no difficulty in dividing the compounds into diuretic or nondiuretic groups. No attempt was made to establish a dose-response relationship although the effects of readily available drugs (ouabain, strophanthidin and digitoxin) were studied in several experiments at doses of from 0.5 to 3.0 mg. Diuretic response increased in rough proportion to dose.

Figure 1 illustrates an experiment in which 0.85 mg of the aglycone strophanthidin was infused during 92 minutes into one renal artery. The dotted vertical lines denote the duration of this infusion. A unilateral diuresis from the infused kidney was evident within 60 minutes. Sodium excretion increased from control period values of 360 μEq per minute to a maximum of 1,360 μEq per minute. At the peak of the diuresis only 74 per cent of the filtered sodium was reabsorbed. Sodium excretion diminished within 10 minutes of cessation of strophanthidin infusion. Changes in urine flow reflected those of sodium excretion increasing from the preinfusion rate of 2.9 to 9.7 ml per minute at the diuretic peak.

Potassium excretion was first reduced, but then increased toward the end of the period of strophanthidin infusion. Unlike sodium, potassium

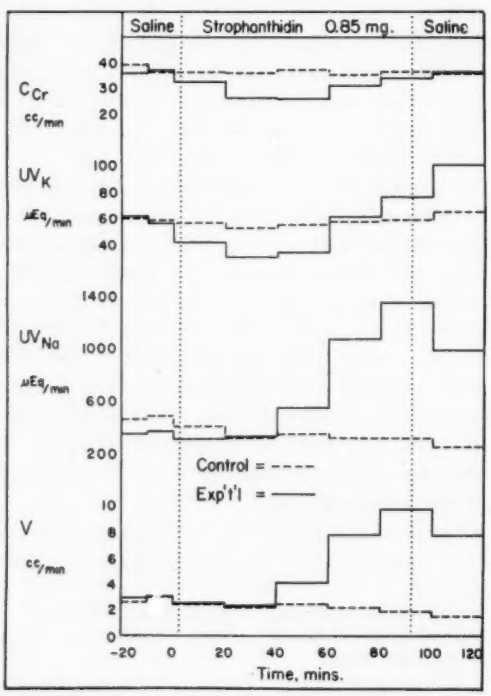

FIG. 1. THE EFFECTS OF UNILATERAL, INTRARENAL ARTERIAL INFUSION OF 0.85 MG OF STROPHANTHIDIN.

[1] We wish to thank Prof. T. Reichstein of Basle, Dr. K. K. Chen, Director of Pharmacological Research, Eli Lilly & Co., Indianapolis, Ind., and Hoffmann-LaRoche Inc., Nutley, N. J., for the steroids used in this work.

excretion continued to increase during the post-infusion period. Creatinine clearance was depressed by strophanthidin but gradually increased as the infusion of the aglycone was continued. PAH clearances, not shown in this figure, paralleled changes in creatinine clearances. The filtration fraction remained constant.

Dihydrostrophanthidinic acid differs from strophanthidin chiefly in the saturation of its lactone ring (see below). As illustrated in Figure 2, this steroid, at a dose 20 times that of strophanthidin, failed to alter any of the renal functions studied.

Table I summarizes the data obtained with the steroids that produced a diuresis. The data from the control and experimental kidneys are presented as average values of two initial clearance periods and of two periods at the time of maximal diuretic action. Included in this table are the experiments at the dosage of the steroid that produced maximal diuresis with minimal depression of PAH and creatinine clearances. The total dose is listed to the left of the table under the names of the compounds. An increase in urine flow and sodium excretion occurred in each instance within 20 to 40 minutes of administration of the steroids. Maximal diuretic activity was achieved in 70 to 80 minutes with ouabain, strophanthidin and emicymarin. The peak of diuresis with digitoxin occurred in approximately 100 minutes and was delayed to about 200 minutes with scillaren-A. This latter compound was the most potent diuretic at the low dosage of 0.5 mg.

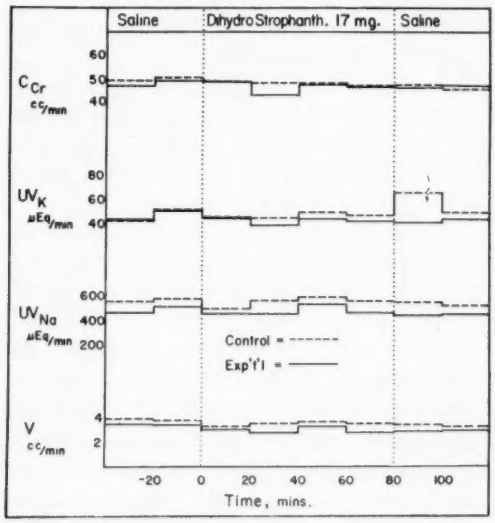

FIG. 2. THE EFFECT OF UNILATERAL, INTRARENAL ARTERIAL INFUSION OF 17 MG OF DIHYDROSTROPHANTHIDINIC ACID. See text.

TABLE I

Summary of results obtained with five steroids that increased urine flow and sodium excretion *

		V cc/min		C_{Cr} cc/min		C_{PAH} cc/min		UV_{Na} μEq/min		UV_K μEq/min	
		Cont	Exp'l	Cont	Exp'l	Cont	Exp'l	Cont	Exp'l	Cont	Exp'l
Digitoxin 2.1 mgs	Initial	4.4	4.5	43	43	130	130	450	400	66	64
	Max	1.4	6.0	33	35	75	84	360	720	62	76
Ouabain 0.9 mgs	Initial	2.3	1.9	36	34	100	100	410	310	49	46
	Max	1.4	9.1	37	26	75	58	210	1300	40	77
Strophanthidin 0.9 mgs	Initial	2.8	2.9	38	37	91	87	470	320	60	59
	Max	2.0	8.7	36	33	87	77	320	1200	59	69
Emicymarin 1.0 mgs	Initial	3.2	3.3	47	47	150	150	490	510	38	51
	Max	1.0	9.7	36	37	90	110	110	1380	14	55
Scillaren-A 0.5 mgs	Initial	1.6	1.3	46	44	120	120	290	260	22	16
	Max	0.7	9.3	40	44	120	110	96	1380	8	60

* See text for mode of presentation of data.

Typically, both creatinine and PAH clearance rates were proportionally diminished soon after the infusions were begun. This reduction of clearances often occurred on the control side as well as on the experimental side. Maximal depression of these clearance rates often preceded maximal diuresis, and recovery occurred on cessation of steroid infusion.

Potassium excretion was variable, although most often it increased concomitantly with sodium excretion. No attempt was made in these studies to obtain further information on alterations in potassium excretion through alterations in experimental protocol.

Table II summarizes the data obtained with steroids which had no effect on the renal excretion of sodium. These compounds were studied in at least two experiments, once at a dose comparable to their active analogs and once at a dose at least eight times greater. Only those experiments in which the larger dose was infused are tabulated. As in Table I, the data are presented for both the control and experimental kidneys. The figures are averages of two initial clearance periods and of two

TABLE II

Summary of results obtained with four steroids that had no effect on urine flow or sodium secretion

		V cc/min		C_{Cr} cc/min		C_{PAH} cc/min		UV_{Na} μEq/min		UV_K μEq/min	
		Cont	Expt'l	Cont	Expt'l	Cont	Expt'l	Cont	Expt'l	Cont	Expt'l
Isostrophanthidin 17 mgs	Initial	2.0	1.7	34	34	89	87	380	320	56	51
	Max	1.9	1.5	38	36	100	93	340	260	49	41
Dihydrostrophanthidinic Acid 17 mgs	Initial	3.9	3.5	51	48	160	160	570	490	48	48
	Max	3.6	3.8	48	47	140	140	560	460	55	42
17α-Emcymarin 8.0 mgs	Initial	1.9	1.7	55	54	180	180	370	360	62	70
	Max	1.8	2.1	58	58	180	210	250	280	15	19
Hexahydro-scillaren 7.5 mgs	Initial	1.8	2.0	42	45	120	130	330	350	51	54
	Max	0.8	1.0	44	45	110	110	90	130	24	29

periods corresponding to the time of maximal activity of their structurally related analogs. In these and other experiments with these compounds, no significant alteration was noted in any of the studied functions.

Table III summarizes the major structural features of the nine steroids under study. At the bottom of the table is a cyclopentanophenanthrene nucleus numbered at the sites of substitution. To the right are the two forms of the C-17 lactone rings. This table is divided on the basis of diuretic activity. The inactive compounds are listed to the right of the corresponding active steroids.

TABLE III

Major structural features of the steroid compounds used in this study *

Diuretic Compounds						Non-diuretic Compounds						
Name	C_3	C_5	C_{10}	Lactone		Name	C_3	C_5	C_{10}	Lactone		
				#	Δ					#	Δ	
Digitoxin	sugar	-H	$-CH_3$	5	α-β							
Ouabain	sugar	-OH	$-CH_2OH$	5	α-β							
Strophanthidin	-OH	-OH	-CHO	5	α-β	Isostrophanthidin	-OH	-OH	-CHO	5		O-bridge C_{14}
						Dihydrostroph. Acid	-OH	-OH	-COOH	5		None
Emcymarin	sugar	-OH	$-CH_3$	5	α-β	17α-Emcymarin	sugar	-OH	$-CH_3$	5		α-β
Scillaren-A	sugar	Δ4-5	-H	6	α-β γ-δ	Hexahydro-Scillaren	sugar	Δ4-5	-H	6		None

* The presence of a double bond between C-4 and C-5 in hexahydro-scillaren-A is in some doubt.

A sugar at C-3 provides favorable solubility and distribution properties, but its presence is not essential for cardiac activity (2–4). The aglycone strophanthidin possesses cardiac (2) and direct diuretic activity.

DISCUSSION

A wide variety of substitutions at the C-5 and C-10 positions is possible within that group of steroids having activity on the heart (4) and on ion flux in red cells (5–9). As is evident from Table III, C-5 and C-10 substitutions of the diuretic compounds do not distinguish them from the nondiuretic compounds. The structure-activity relationship is most evident at the C-17 substitution. The lactone ring must be β oriented at this site. The single example of α orientation in this series is represented by the nondiuretic compound 17α-emicymarin. Whether 5 or 6 membered, the lactone ring of the diuretic steroids are unsaturated in the α, β position. Activity is lost with saturation of the ring as typified by dihydrostrophanthidinic acid and hexahydro-scillaren-A. The unsaturation of the lactone ring is unstable when shifted to the β, γ position. In this state, an oxygen bridge forms from the γ carbon of the lactone ring to the C-14 hydroxyl group (23). Strophanthidin is transformed by this route into the inactive "iso" compound.

We conclude then, that the most specific constituent associated with diuretic activity is the lactone ring. Diuresis was observed solely with those steroids having a C-17, β oriented lactone ring unsaturated in the α, β position.

The molecular similarity of the digitalis steroids to the mineralocorticoids suggests that they might produce diuresis by an "anti-aldosterone effect." From the present study, evidence against anti-aldosterone activity of this group of steroids lies in the magnitude of their action on sodium reabsorption. Aldosterone and other mineralocorticoids are believed to stimulate the reabsorption of no more than about 2 to 3 per cent of the filtered sodium load (22, 24). In these studies as well as in those of Orloff and Burg (18), 15 to 40 per cent of the filtered sodium appeared in the

urine subsequent to the administration of the digitalis steroids.

The evidence to date points to an inhibition of ion carrier mechanisms as the mode of action of these compounds, although the possibility of interference of energy supply to ion transport cannot be completely excluded (9). In red cell suspensions digitalis does not alter glycolysis or oxygen uptake (5). Likewise, organic phosphate levels and adenosine triphosphate resynthesis are not affected (25). These observations favor the hypothesis of carrier mechanism interference. Added to these are the observations of both Glynn (9) and Schatzmann (5) who report kinetic alterations studied in red cell suspensions highly suggestive of competitive inhibition for carrier states. Glynn noted that the cardiac steroids inhibit certain passive diffusion fluxes, thus suggesting a direct effect on the carrier mechanism rather than interference with energy supply. Translating this hypothesis to the kidney has its dangers, but in view of the remarkable structural specificity of the compounds common to activity in all three tissues, we suggest that ion carrier inhibition is also the mode of their renal action.

SUMMARY

Nine digitalis steroids have been studied with respect to diuretic properties. These compounds were administered to dogs in acute experiments by infusion into one renal artery. Ureteral urines were collected separately.

Five of these steroids, known to affect cardiac activity and cation movement in *in vitro* preparations, increased sodium and water excretion from the infused kidney. This effect was independent of changes in renal hemodynamics. The dose necessary to produce diuresis ranged from 0.5 to 2.1 mg. In optimal quantity, these compounds blocked the reabsorbtion of 35 per cent of the filtered sodium.

Four of the steroids failed to increase sodium or water excretion in doses up to 17 mg. These latter compounds are those which lack significant cardiac activity or inhibitory action on cation movement *in vitro*.

The biologically active compounds are further distinguished by their chemical structure. Activity on the heart, kidney and on *in vitro* ion fluxes is most closely associated with the following structural criteria: a 5 or 6 membered lactone ring attached in β orientation to C-17 of the steroid nucleus, unsaturated between the α and β carbons. The probable mode of action is inhibition of a carrier mechanism.

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THE PULMONARY BLOOD VOLUME IN MAN *

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The measurement of blood volume in the pulmonary vascular bed during life became theoretically feasible with the introduction of indicator dilution methods by Stewart (1) and Hamilton, Moore, Kinsman and Spurling (2). The principles and formulas set forth by these investigators have since undergone extensive scrutiny by theoretical analysis, in circulation models, and *in vivo*. As a result of these studies, there exists now general agreement that the introduction of an indicator substance into the central circulation, either directly or by peripheral venous injection, and downstream recording of its concentration change with time during the first transit permits the determination of three important circulatory parameters: *1*) the cardiac output, *2*) the mean transit time from injection to sampling site, and *3*) by multiplication of cardiac output and mean transit time, the circulating volume of blood between injection and sampling sites, including all temporally equidistant points in the vascular bed.

Practically speaking, then, the measurement of pulmonary blood volume *in vivo* requires determination of the mean transit time of an indicator from the pulmonary artery to the left atrium together with cardiac output.

The development of right and left heart catheterization has made the pulmonary artery and left atrium accessible as injection and sampling sites in man. Injection of an indicator through a cardiac catheter into the pulmonary artery is easy. The recording of dilution curves by sampling from the left atrium is feasible but cumbersome. Furthermore, it is uncertain whether an indicator injected into the pulmonary artery and sampled from the left atrium will undergo complete mixing, since it does not traverse a ventricular chamber in which turbulent flow exists.

In the present study, an attempt was made to measure pulmonary blood volume in man by the Stewart-Hamilton method and yet avoid left atrial sampling and the uncertainty of adequate mixing in the pulmonary circuit. The procedure employed was the simultaneous injection of two different indicators into the pulmonary artery and left atrium, with sampling from the brachial artery. This yields duplicate determinations of cardiac output as well as the mean transit time of indicator A from the pulmonary artery to the brachial artery and of indicator B from the left atrium to the brachial artery. By subtraction, the mean transit time from pulmonary artery to left atrium is obtained, and pulmonary blood volume can be calculated. Both indicators traverse the left ventricle where they are exposed to a common degree of turbulent mixing. The use of a common collecting system ensures that any distortion of the time-concentration curves due to its particular properties would affect both indicators equally and thus, in effect, cancel out. The principle of the determination is represented schematically in Figure 1.

Since the method requires simultaneous catheterization of the right and left heart, no observations in normal subjects were carried out. The determinations were made in the course of diagnostic studies on 45 adult patients who had or were suspected of having mitral or aortic valvular disease of sufficient complexity to warrant combined right and left heart catheterization for evaluation of their cardiac status.

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SCHEMA OF CIRCULATION TO ILLUSTRATE DETERMINATION OF PULMONARY BLOOD VOLUME

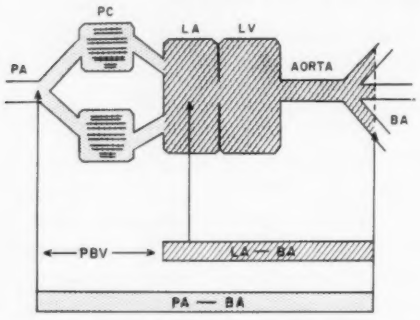

FIG. 1. TWO INDICATORS ARE INJECTED SIMULTANEOUSLY INTO PULMONARY ARTERY (PA) AND LEFT ATRIUM (LA) AND SAMPLED FROM BRACHIAL ARTERY (BA). The difference between the two mean transit times, multiplied by the cardiac output, equals the pulmonary blood volume (PBV). See text for details.

MATERIAL AND METHODS

Pulmonary blood volume was determined in 45 patients, ranging in age from 22 to 60 years, average age, 41 years. There were 19 males and 26 females, none of whom had congenital heart disease or circulatory shunts. In most instances, combinations of valvular lesions were present. The classification employed throughout this report groups the patients according to the defect which was considered the hemodynamically preponderant one on the basis of clinical picture, catheterization findings and, wherever applicable, subsequent surgical observations. By these criteria, 15 patients had predominant mitral stenosis, 13 predominant mitral regurgitation, 7 predominant aortic stenosis, 3 predominant aortic regurgitation. There were 3 patients with equally significant mitral and aortic valve disease; of these 1 had stenosis of both valves, 1 had both mitral and aortic regurgitation, and 1 had aortic stenosis and mitral regurgitation. Four patients had no demonstrable hemodynamic derangement. The final diagnoses in these were coronary artery disease in 1, restrictive pleuritis in 1, and no demonstrable cardiac or pulmonary abnormality in 2. This group of 4 patients will be referred to subsequently under the heading "normal circulatory dynamics."

All patients were studied in the fasting state, premedicated with 100 mg secobarbital orally and 50 to 75 mg meperidine intramuscularly. In some instances, an additional 8 mg morphine sulfate was given intramuscularly just before the left heart catheterization. A venous catheter (size 6 or 7F, 125 cm in length) was introduced under local procaine anesthesia via the right antecubital vein, and its tip placed in the right or left main pulmonary artery, just beyond the bifurcation of the pulmonary trunk. The left brachial artery was cannulated with a no. 16 gage Cournand needle. The patient was then turned to the prone position and left heart catheterization performed by the percutaneous paravertebral route as previously described (3), introducing a no. 16 gage thin-walled needle into the left atrium. A 40 cm length of PE60 polyethylene tubing was passed through the lumen of the needle, advanced across the mitral valve for pressure measurements in the left ventricle, and then withdrawn until its tip rested in the left atrium. Pressures were recorded through Statham P23D strain gage manometers on a Sanborn Polyviso direct-writing oscillograph. The zero point for all pressure measurements was taken to be 10 cm anterior to the skin of the back at the level of the seventh thoracic vertebra. Immediately after the simultaneous recording of pressures in the pulmonary artery, left atrium and brachial artery, the indicator dilution studies were carried out. Upon a prearranged signal, simultaneous rapid injections were made of 3 ml Evans blue dye into the pulmonary artery and 3 ml I^{131}-labeled human serum albumin (6 to 8 μc per ml) into the left atrium, while blood was allowed to flow from the arterial needle through a 28 cm length of PE280 polyethylene tubing (volume 1.0 ml) into a rotating wheel-type fraction collector containing test tubes with a few crystals of dried heparin. Each tube collected blood for exactly 2 seconds. The time and duration of the left atrial injection and the passage of the sampling tubes were inscribed on the Polyviso record via electric relay switches. The recorded duration of the injection was usually 1 second; the midpoint of the injection period was considered zero time for all transit time measurements. Gibson syringes were used for all indicator injections; the amount delivered from syringe and catheter was calibrated by weight. After completion of the procedure, all sample tubes were centrifuged, and 0.5 or 1.0 ml aliquots of undiluted plasma analyzed for radioactivity in a well-type scintillation counter for sufficient time to ensure less than 1 per cent error. In some instances during earlier experiments, the radioactivity determinations were performed on similar aliquots of whole blood pipetted from the sample tubes after gentle agitation prior to centrifugation. Dye concentration in the undiluted plasma was then determined on the same specimens in a Beckman DU spectrophotometer using Pyrocell microcuvets, reading extinction at 620 mμ against a blank of the patient's own plasma. Standards of the radioactive injectate were prepared in quadruplicate by 1:1,000 dilution in normal saline. Dye standards were prepared by quantitative dilution of the injectate in each patient's own plasma. Aliquots of the injectate standards were analyzed for radioactivity and dye concentration in the same manner as the samples. Hematocrits were determined in quadruplicate on samples from the fraction collector immediately preceding and following the appearance of the indicator curves, by spinning with a centrifuge radius arm of 19 cm for 30 minutes at 3,000 rpm in Wintrobe tubes, and corrected for trapped plasma as advocated by Gregersen (4). Time-concentration curves were plotted on semilogarithmic graph paper for

each indicator and extrapolated through three logarithmic cycles from the onset of the straight-line downslope. Cardiac output and mean transit time were calculated from each curve by the usual Stewart-Hamilton formulas (1, 2) as follows:

$$\text{C.O.} = \frac{(60)(\text{Vol. I})(\text{Conc. I})}{(\Sigma\ \text{Conc.})(1 - \text{Hct})}$$

$$\text{MTT} = \frac{\Sigma(\text{Conc.} \times \text{Time})}{\Sigma\ \text{Conc.}}$$

where

C.O. = cardiac output (L/min)
MTT = mean transit time (sec)
Vol. I = volume of indicator injected (ml)
Conc. I = concentration of indicator injected
Σ Conc. = sum of the concentrations read at 1-second intervals from the indicator dilution curve, extrapolated through 3 logarithmic cycles
Time = time at which each concentration occurred, in seconds, from midpoint of injection period
Hct = hematocrit, corrected for trapped plasma = observed Hct × 0.96.

Delay in the collecting system was calculated as follows:

$$\text{delay} = \frac{\text{collecting system volume (1.0 ml)}}{\text{rate of flow (ml/sec)}}.$$

The calculated delay was subtracted from all mean transit time measurements.

The average of the two cardiac output measurements as calculated from the I^{131} curve and from the Evans blue dye curve was used for all volume calculations except for a few instances in which a part of either the radioactive iodine or the dye was lost from the syringe during the process of injection. These situations do not involve an error in the mean transit time calculation for that indicator. The following volume calculations were carried out:

$$\text{PA} \rightarrow \text{BA volume} = \text{C.O. (ml/sec)} \times \text{MTT PA} \rightarrow \text{BA (sec)}$$

$$\text{LA} \rightarrow \text{BA volume} = \text{C.O. (ml/sec)} \times \text{MTT LA} \rightarrow \text{BA (sec)}$$

$$\text{Pulmonary blood volume} = \text{C.O. (ml/sec)} \times (\text{MTT PA} \rightarrow \text{BA} - \text{MTT LA} \rightarrow \text{BA})\text{(sec)}.$$

RESULTS

The results of the study are listed in Table I, together with the pertinent hemodynamic parameters.

Cardiac output

There was substantial agreement between the simultaneous cardiac output determinations, as shown in Figure 2. The average ratio of the flows as calculated from left atrial and pulmonary arterial injections was 0.98, with a standard deviation, computed from the distribution of ratios, of 0.15.

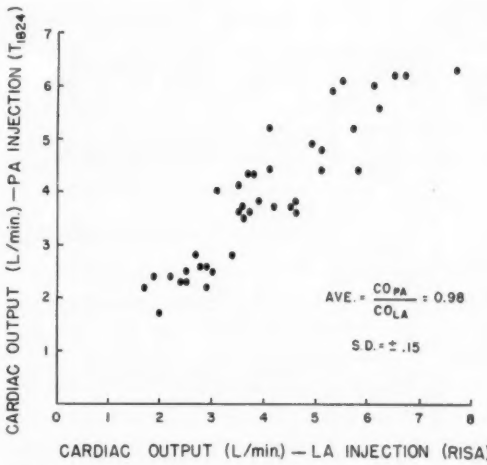

FIG. 2. SIMULTANEOUS CARDIAC OUTPUTS OBTAINED BY INDICATOR DILUTION TECHNIQUE, USING T-1824 INJECTED INTO PULMONARY ARTERY AND RADIO-IODINATED SERUM ALBUMIN (RISA) INJECTED INTO LEFT ATRIUM.

No systematic differences between the two determinations were found, although there were occasional divergences for which no obvious explanation could be offered.

Mean transit time

Pulmonary mean transit time. The mean transit time from pulmonary artery to left atrium varied widely, between the extremes of 3.2 and 28.0 seconds [1.9 and 15.2 seconds per m^2 body surface area (BSA)], with an average of 9.1 seconds (5.6 per m^2) for the entire group. It tended to be more prolonged in the patients with mitral valve disease—average 10.7 seconds (6.4 per m^2) in the mitral stenosis group; 9.2 seconds (5.9 per m^2) in the mitral regurgitant group; and 9.6 seconds (6.2 per m^2) in the three patients with combined mitral and aortic disease. The mean for the patients with aortic valve disease was 7.8 seconds (4.8 per m^2) and for the group with normal circulatory dynamics, 5.3 seconds (3.5 per m^2). Much overlap of values was evident among the different groups.

Mean transit time from left atrium to brachial artery. With the exception of five outstandingly prolonged values in patients with severe mitral regurgitation (18.4 to 34.5, 13.0 to 24.5 seconds per m^2), the transit times between left atrium and brachial artery ranged from 3.1 to 14.8 seconds

TABLE 1

Hemodynamic data

Name	Age	Sex	BSA	Cardiac output PA injection	Cardiac output LA injection	Average cardiac index	MTT PA-BA	MTT LA-BA	MTT PA-LA	Blood volume PA-BA	Blood volume LA-BA	Pulmonary blood volume, PA-LA	Mean pressure PA	Mean pressure LA	Pulmonary vascular resistance	Left atrial size (X-ray*)
			m²	*L./min*		*L./min/m²*	*sec*	*sec*	*sec*	*ml/m²*	*ml/m²*	*ml/m²*	*mm Hg*		*dynes-sec-cm⁻⁵*	
Normal circulatory dynamics																
E.K.	42	F	1.68	5.2	4.1	2.7	9.1	4.3	4.8	415	196	219	14	7	122	N
J.P.	54	M	1.76	6.3	7.7	4.0	9.8	6.2	3.6	648	411	239	16	6	114	N
F.F.	44	M	1.99	6.2	6.5	3.2	12.9	8.1	4.8	691	434	257	16	8	100	1+
W.H.	60	M	1.98		4.1	2.1	15.0	7.2	7.8	518	248	269	20	6	273	N
Predominant mitral stenosis																
C.K.	26	F	1.56	4.4	4.1	2.7	8.8	4.5	4.3	395	202	193	18	7	209	1+
M.B.	38	F	1.75	3.6	4.6	2.3	11.5	6.4	5.1	449	250	199	19	9	195	1+
G.H.	45	F	1.65	3.7	3.6	2.2	14.6	7.6	7.0	545	284	262	36	30	130	2+
P.M.	47	F	1.42		3.7	2.6	13.0	6.8	6.2	565	295	269	30	19	238	2+
M.Mc	36	F	1.53	2.5	3.0	1.9	18.6	9.6	9.0	567	293	275	32	20	343	2+
T.F.	41	M	1.89	1.7	2.0	1.0	29.2	12.1	17.1	489	203	287	59	27	1,346	2+
R.M.	44	F	1.57	5.6	6.2	3.8	7.8	3.1	4.7	489	194	294	28	18	135	1+
E.F.	47	F	1.57	2.3	2.5	1.5	21.6	9.1	12.5	550	232	318	94	40	1,798	2+
J.H.	30	F	1.37	2.8	2.7	2.0	18.9	9.1	9.8	621	299	322	85	33	1,539	2+
F.F.	45	M	1.66	4.3	3.8	2.5	16.4	8.5	7.9	675	350	325	68	30	741	2+
C.D.	42	F	1.56	3.6	3.7	2.4	13.1	4.7	8.4	518	186	332	61	36	540	2+
W.P.	43	F	1.47	3.7	4.5	2.8	14.2	6.9	7.3	660	321	339	25	21	78	2+
B.R.	30	F	1.82	4.0	3.1	2.0	25.2	10.8	14.4	831	356	475	94	40	1,199	2+
O.G.	46	M	1.83		3.0	1.6	32.9	14.8	18.1	899	404	495	28	22	160	2+
J.Z.	48	M	1.84	2.5	2.5	1.4	40.9	12.9	28.0	926	292	634	36	23	416	2+
Predominant mitral regurgitation																
M.G.	45	F	1.67	4.1		2.5	9.5	5.3	4.2	389	217	172	66	21	877	2+
M.C.	44	F	1.41	2.2	1.7	1.4	42.8	34.5	8.3	1,012	816	196	47	32	599	4+
I.R.	27	F	1.32	4.1	3.5	2.9	11.0	6.2	4.8	527	298	230	13	9	84	1+
A.E.	38	M	1.92	2.3	2.4	1.3	32.0	19.4	12.6	667	404	263	51	31	666	3+
J.R.	36	F	1.48	3.8	3.9	2.6	12.3	5.8	6.5	541	255	286	14	5	184	2+
M.C.	38	F	1.37	2.6	2.8	2.0	27.5	18.6	8.9	904	611	293	49	27	651	3+
E.H.	35	F	1.56	6.0	6.1	3.9	9.7	4.3	5.4	622	276	346	16	6	133	2+
McQ.	49	F	1.26	2.6	2.9	2.1	39.2	29.1	10.1	1,400	1,039	361	39	19	592	3+
G.P.	27	F	1.42	2.2	2.9	1.8	30.7	18.4	12.3	901	540	361	24	15	288	3+
H.T.	60	M	1.90		3.2	1.7	28.3	13.8	14.5	794	387	407	56	21	874	2+
A.C.	45	F	1.52	3.7	4.2	2.6	19.6	10.0	9.6	860	432	421	29	25	80	3+
R.B.	44	M	2.07	4.4	5.1	2.3	21.1	7.8	13.3	799	295	503	39	22	289	2+
M.S.	45	F	1.55	4.4	5.8	3.3	18.0	8.5	9.5	987	466	521	24	19	78	3+
Predominant aortic stenosis																
J.D.	56	M	1.80	3.5	3.6	2.0	17.8	12.1	5.7	577	392	185	19	12	160	N
A.P.	51	M	1.78	6.1	5.5	3.3	11.8	7.7	4.1	641	418	223	16	13	41	2+
S.S.	41	M	1.68	4.9	4.9	2.9	12.5	7.9	4.6	608	384	224	21	13	130	2+
K.W.	31	M	1.82	4.8	5.1	2.7	12.9	5.6	7.3	579	251	328	17	7	163	N
V.D.	45	F	1.50	3.8	4.6	2.8	12.3	5.0	7.3	574	233	341	9	1	1[F]2	N
J.W.	34	M	1.58	3.6	3.4	2.3	16.4	5.9	10.5	623	224	399	23	8	3[3]	N
L.P.	22	M	1.62	4.3	3.7	2.5	20.6	10.3	10.3	848	424	423	38	22	320	2+
Predominant aortic regurgitation																
N.W.	35	F	1.66	5.2	5.7	3.3	7.8	4.6	3.2	431	254	177	11	3	116	N
W.P.	41	M	1.65	6.2	6.7	3.9	20.5	14.3	6.2	1,325	924	401	50	22	350	2+
J.L.	39	M	1.86	2.8	3.4	1.7	33.7	14.7	19.0	936	409	527	52	15	954	2+
Combined mitral and aortic lesions																
T.D.	45	F	1.47	2.4	2.2	1.6	18.5	11.2	7.3	482	292	190	21	15	209	2+
J.B.	36	M	1.90	5.9	5.3	3.0	15.3	8.3	7.0	752	408	344	24	13	157	2+
L.F.	39	F	1.48	2.4	1.9	1.5	24.1	9.6	14.5	597	238	359	63	23	1,453	2+

* Arbitrary assessment of size on basis of normal (N) to 4+.

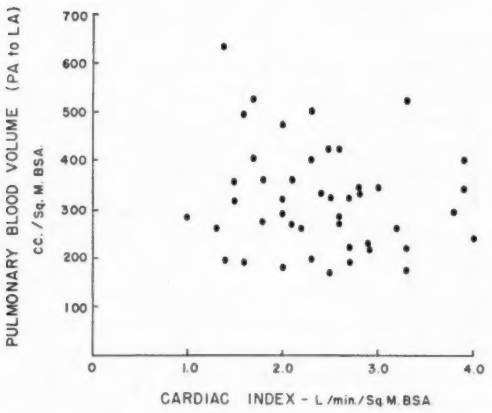

FIG. 3. RELATIONSHIP BETWEEN CARDIAC OUTPUT AND PULMONARY BLOOD VOLUME. There is no evident correlation between pulmonary blood flow and pulmonary blood volume.

(2.0 to 8.1 per m^2), with no notable differences between the different groups. In the four patients with normal circulatory dynamics, the range was 4.3 to 8.1 seconds (2.6 to 4.1 per m^2).

Pulmonary blood volume

The pulmonary blood volume was found to range from 172 to 634 ml per m^2 BSA, with a mean of 322 ml per m^2 for the entire group. The patients with mitral valve disease tended to show slightly higher values than the remainder, the mean for the mitral stenosis group being 335, for mitral regurgitation 335, for combined mitral and aortic disease 298, and for all patients with mitral disease 331 ml per m^2 as against 323 ml for those with aortic disease alone and 246 for the subjects with normal circulatory dynamics. There was much overlap among the groups, and the sample of patients without mitral disease was too small to assign statistical significance to the difference between the means.

Volumes between left atrium and brachial artery

Four patients with mitral regurgitation and one with aortic regurgitation were found to have marked increases of blood volume, relative to the other cases, in the compartment which includes the chambers of the left heart and portions of the proximal arterial tree. The calculated volumes in these five patients ranged from 540 to 1,039 ml per m^2 BSA, and all showed marked enlargement of the left atrium and ventricle by X-ray. All remaining patients fell in a range of volumes from 186 to 466 ml per m^2, without significant differences between the different groups. In general, the pulmonary blood volume and the volume between left atrium and brachial artery tended to be of approximately equal size.

Correlations

Possible correlations between the calculated pulmonary blood volume and various simultaneously measured hemodynamic parameters were examined. Since pulmonary blood volume is the product of cardiac output and mean transit time, a correlation with one or the other of these two factors is inherent in the calculation. No correlation with cardiac output was found (Figure 3), but higher pulmonary blood volumes were associated with longer pulmonary mean transit times (Figure 4) ($R = +0.769$). There was also an inverse relationship between cardiac index and pulmonary mean transit times (Figure 5). The correlation coefficient, utilizing the logarithmic transformation of the pulmonary mean transit time, was -0.742. No correlation was found between pulmonary blood volume and pulmonary vascular resistance or pressure difference across

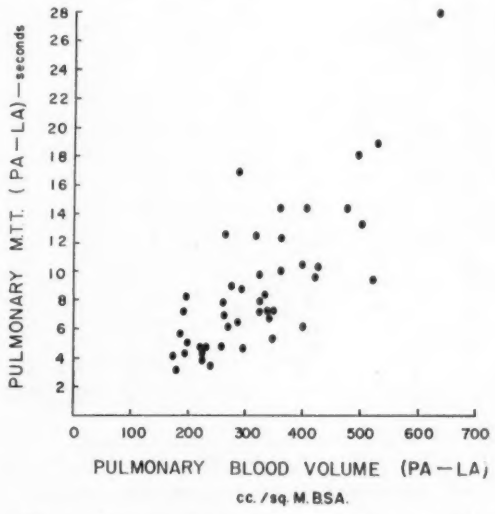

FIG. 4. RELATIONSHIP BETWEEN PULMONARY BLOOD VOLUME AND PULMONARY MEAN TRANSIT TIME. A significant direct correlation is seen between pulmonary blood volume and mean transit time, i.e., the pulmonary blood volume varies inversely with the velocity of blood flow.

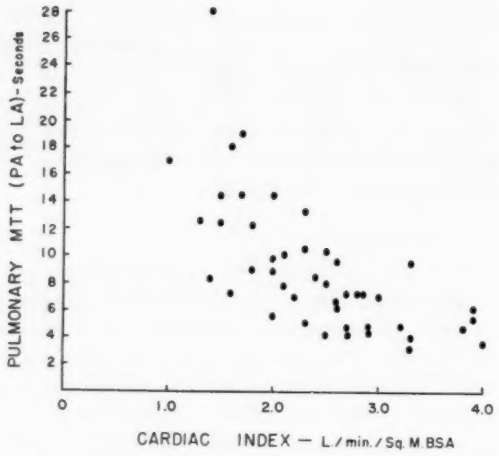

FIG. 5. RELATIONSHIP BETWEEN CARDIAC INDEX AND PULMONARY MEAN TRANSIT TIME. There is a direct correlation between pulmonary blood flow and velocity.

the pulmonary circulation (pulmonary arterial mean minus left atrial mean pressures). Figures 6 and 7 show plots of pulmonary blood volume against pulmonary arterial mean pressure and left

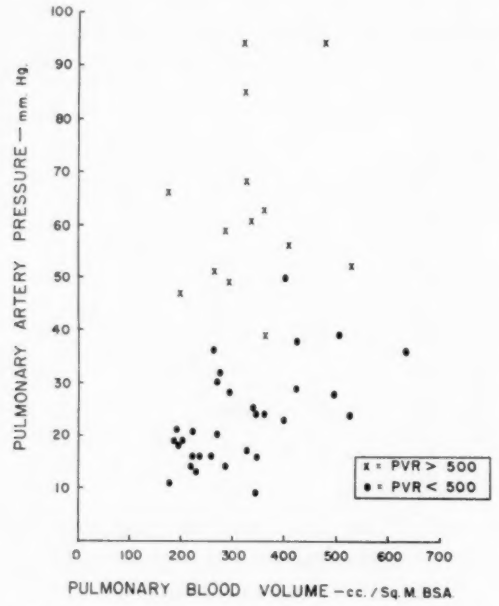

FIG. 6. RELATIONSHIP BETWEEN PULMONARY ARTERY PRESSURE AND PULMONARY BLOOD VOLUME. There is a correlation between the volume of blood in the lungs and the pulmonary arterial pressure, which is most evident in those cases without marked elevation of pulmonary vascular resistance. See text for statistical significance.

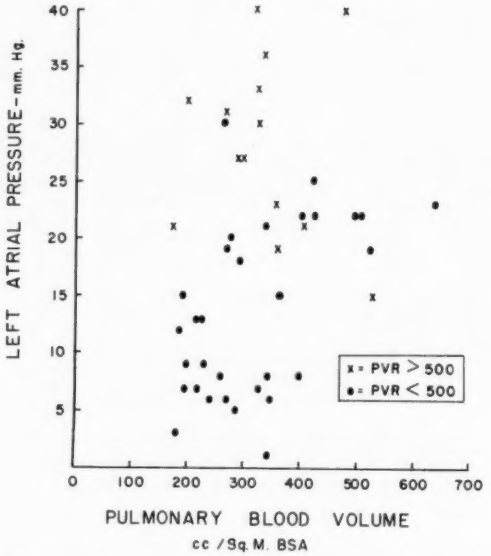

FIG. 7. RELATIONSHIP BETWEEN LEFT ATRIAL PRESSURE AND PULMONARY BLOOD VOLUME. An apparent correlation exists between left atrial pressure and pulmonary blood volume, particularly in the low resistance group.

atrial mean pressure. If all cases are included, no significant correlation exists, the R values being 0.258 and 0.283, respectively. On the other hand, if patients with pulmonary vascular resistances above and below 500 dynes-sec-cm^{-5} are considered separately, as in Figures 6 and 7, a tendency for increased pressures to be associated with increased volumes becomes apparent as long as the vascular resistance remains below 500 dynes-sec-cm^{-5}. Excluding the cases with resistances above this level, the correlation coefficients were 0.582 and 0.524, respectively, both being highly significant ($p < 0.001$). Those patients with severely elevated pulmonary vascular resistances tended to have lower volumes than might have been expected from the pressure-volume relationship in the low resistance group.

There was no evident correlation between the volume of the pulmonary vascular compartment and the age, sex or BSA of these adult patients.

DISCUSSION

Consideration of possible errors of the method

The validity of measuring pulmonary blood volume by the method employed rests on certain assumptions.

1. Proportionality of indicator to flow and velocity of flow through the two lungs. Either the indicator injected into the pulmonary artery must be equally distributed to the two lungs, or if it is not equally distributed, the velocity of flow through the two lungs must be identical. There is no reason to believe that indicator injected into the main pulmonary artery or into one branch becomes equally distributed in each main branch. In fact, the probability is that it does not. If the bolus of indicator is diverted predominantly to one lung, the pulmonary mean transit time affords calculation of the blood volume of that lung plus the volume of temporally equidistant vessels of the opposite lung. For the true blood volume of the latter to be included, it is necessary to assume equal velocity of blood flow through both lungs. This assumption has not been tested.

2. Bronchial circulation. The bronchial circulation anastomoses with the pulmonary circulation at the arterial, capillary, and venous levels (5). The flow is said to amount to between 2 and 5 per cent of the cardiac output in normal man. The temporally equidistant portions of these bronchial collaterals entering the lung are included in both the pulmonary arterial and left atrial injection volumes and therefore cancel out when these two are subtracted from one another to arrive at the calculation of pulmonary blood volume. If, however, blood passes from pulmonary artery to the bronchial system as a result of a higher pressure in the pulmonary than in the corresponding systemic circulation, the calculated pulmonary blood volume would be artifactually large, due to an inclusion of this right to left volume which would not be included in the left atrial to brachial arterial volume. It seems unlikely that such a pulmonary to bronchial shunt would be of important magnitude.

3. Mixing of indicator in left atrium. In the event of incomplete mixing of indicator injected into the left atrium, the calculated pulmonary blood volume would include that portion of the chamber volume in which the left atrial injectate did not mix and would thus be falsely high. Evidence indicates that there may indeed be incomplete mixing of indicator within the left atrium, and the extent of incomplete mixing would presumably be dependent upon the indicator's being uniformly distributed in all parts of the atrium, and this would be affected in part by the time of indicator injection relative to the cardiac cycle. The degree to which this mixing factor influences the results of this study cannot be determined at present. Although mixing might be expected to be less adequate with increasing chamber size, there was no direct relationship between the calculated pulmonary blood volume and the radiologic size of the left atrium (Figure 8). The largest left atrium to brachial artery volumes were recorded among the group of cases with predominant mitral regurgitation, which was associated with the greatest degree of left atrial and left ventricular enlargement. Inordinately high values for pulmonary blood volume were not seen in this group. Experimental evidence indicates that regurgitation into a chamber may greatly facilitate the mixing of injectate therein (6).

The possibility exists that the two indicators may not be thoroughly distributed throughout the entire volume of the left ventricle, but it seems reasonable to expect that the "mixing volume" within that chamber is approximately the same for both indicators. Under these conditions, the mean transit time of the two indicators through the ventricle would be the same.

4. Transit time of indicator and whole blood. It is assumed that the transit time of indicator is the

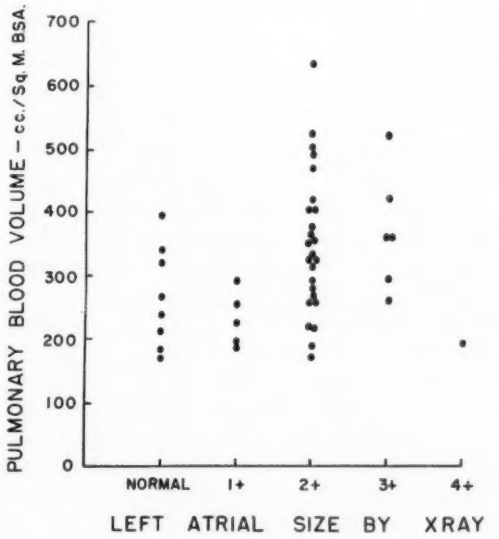

FIG. 8. LEFT ATRIAL CHAMBER SIZE AND PULMONARY BLOOD VOLUME. There is no correlation between the size of the left atrium by X-ray and the calculated pulmonary blood volume.

same as that of whole blood Both the indicators utilized in this study were carried within the plasma fraction. Although there is evidence that the transit time for cells is faster than that for plasma within the central circulation (7), the error introduced into the pulmonary blood volume calculation by this difference is not considered to be significant.

5. Cardiac output. Cardiac output determinations by the indicator dilution method are believed to have an accuracy similar to that of the Fick method, both having an error of about 5 per cent (8, 9). The contribution of technical errors to this percentage should be reduced by obtaining the average of two simultaneous cardiac output determinations with two different indicators. The appearance of recirculating indicator before satisfactory recording of the exponential decay portion of the curve may cause errors in downslope extrapolation which affect both the cardiac output figures and the mean transit time determination in varying degrees. This would be more likely to occur in the dilution curve from pulmonary arterial injection, due to the larger central volume and the longer time required for inscription of the curve. However, the absence of a systematic difference between the pairs of simultaneous cardiac output values, particularly in the range of the very low outputs, suggests that this has not been a source of significant error in these cases (Figure 2). It was necessary to eliminate several cases of severe valvular regurgitation from the study because the prolonged nature of the curves made it difficult to define the exponential decay portion of the curve in the presence of recirculating indicator.

6. Measurement of mean transit time. The timing of the beginning and duration of the two indicator injections was not independently recorded. Injection through the polyethylene catheter into the left atrium requires 0.5 to 1.0 seconds, while injection into the pulmonary artery through the Cournand catheter requires 1.0 to 1.5 seconds. Due to the larger volume of the latter, indicator will not reach the tip of the catheter until a finite length of time after entry of the left atrial indicator into that chamber. The timing of both indicator curves was related to the midpoint of the left atrial injection. This introduces an error in the determination of the mean transit time of the pulmonary arterial indicator, which is assumed not to exceed 0.75 second. The two indicator curves were sampled simultaneously in a fraction collector, thus avoiding a change in the state of the patient. Samples were collected at 2-second intervals, producing a potential error in mean transit time of 1 second. An alternative technique used by McGaff, Jose and Milnor, has been to inject indicators into the pulmonary artery and left atrium consecutively rather than simultaneously and record the time-concentration curve at the brachial artery with a densitometer (10). This has the advantage of potentially improving the accuracy of timing, but the disadvantage of nonsimultaneous determinations. It is currently not possible to record accurately two different dyes simultaneously with two different densitometers.

Because the same aliquots of blood were analyzed for both indicators, errors in the calculated pulmonary mean transit time due to faulty timing of the collecting system, measurement of delay within the sampling catheter, and irregular skewing of the time-concentration curve by the peripheral arterial system have been eliminated.

In considering the above sources of error in the cardiac output and mean transit time determinations, it must be assumed that an over-all error of 20 to 30 per cent may thus be introduced into the calculation of the pulmonary blood volume.

Comparison with previous studies of central blood volume

The circulating blood volume of the pulmonary vascular compartment has defied exact measurement despite Stewart's demonstration in 1897 of the theoretical basis for this calculation (1). Further theoretical and experimental confirmation of the validity of this volume determination, utilizing the product of the mean transit time of indicator particles and the blood flow, has been given repeatedly (2, 11–14).

Investigators have previously calculated "central," "intrathoracic," and "pulmonary" blood volumes by this method. However, the inability to measure the mean transit time of indicator particles traveling from pulmonary artery to left atrium has prevented determination of the pulmonary vascular volume alone. The results of several of the earlier studies are compared with the

TABLE II

Central blood volumes in man *

Compartment	Subjects		Average volume	Average % of total blood volume
	No.	Type		
			ml/m² BSA	
Peripheral vein to systemic artery	91	Normal	1,370	60
Pulmonary artery to systemic artery	76	Normal	650	23
Right ventricle to	46	Normal	690	28
left ventricle	12	Mitral stenosis	609	29
Pulmonary artery to left atrium				
Kunieda (23)	23	Rheum. heart disease	350	12–14†
Milnor and associates (24)	19	Rheum. heart disease	365	12–15†
Present study	45	Rheum. heart disease	322	11–13†

* Stewart-Hamilton method adopted from Lammerant (15).
† Assuming total blood volume = 2.5 to 3.0 L/m² BSA.

volumes found in the present investigation in Table II, with the results grouped according to the vascular compartment measured. This table was adapted from Lammerant (15). The first group of studies (16–20), utilizing peripheral vein injections with sampling from a systemic artery, revealed central volumes averaging 60 per cent of total blood volume comprising the heart, lungs, and large portions of the arterial and venous systems. Pulmonary arterial injections, in the second group, permitted calculation of the blood volume of the lungs, left heart, and part of the systemic arterial compartment, with values averaging 23 per cent of total blood volume (9, 21, 22). Lammerant determined the pulmonary mean transit time from biphasic dilution curves recorded from a precordial surface counter following peripheral vein injection (15). The calculated central volumes, probably including significant portions of the heart chamber volumes, averaged 28 to 29 per cent of the total blood volume.

Kunieda in 1955 (23) and, recently, Milnor, Jose and McGaff (24) have reported values for pulmonary blood volume closely approximating those of the current study, determined by similar methods in comparable groups of patients. The values obtained in these studies are of a significantly lower order of magnitude than those reported by others. Although the total blood volume was not determined, by assuming it to average between 2.5 and 3.0 L per m² BSA (25, 26), the pulmonary blood volume was found to represent approximately 12 per cent of the total blood volume. In the four patients with normal circulatory dynamics, this figure was 9 per cent.

Factors influencing the pulmonary blood volume

A previous report from this laboratory by Rapaport, Kuida, Haynes and Dexter (27) described the general lack of agreement in the literature concerning the extent to which certain factors may influence the volume of the pulmonary vascular compartment. The values obtained in this study, representing what is considered to be a closer approximation to the true pulmonary blood volume, have been correlated with other hemodynamic parameters measured during combined right and left heart catheterization.

Contrary to the findings of Rapaport and coworkers (27), no relationship was noted between cardiac output and pulmonary blood volume. One evident explanation for this divergence of results is that the earlier study contained a smaller number of cases and pertained to the volume of blood between pulmonary artery and brachial artery. The alternate possibility is that the present study included a greater number of patients with severe pulmonary vascular disease, in which case an altered vessel distensibility could obscure a direct flow-volume relationship. No such relationship was evident, however, when the cases exhibiting high pulmonary vascular resistance were segregated from the remainder of the group.

It is reasonable to suspect that pressure eleva-

tion within the left atrium would be reflected by a volume increase in the pulmonary vascular bed and perhaps in the calculated pulmonary blood volume. Indeed, it has been thought that pulmonary "congestion" is responsible for most of the clinical picture associated with mitral valve disease and/or left ventricular failure. A correlation was found in this study between the level of left atrial pressure and pulmonary blood volume, the mean pulmonary blood volume being 277 ml per m^2 in the group of cases with left atrial pressure 15 mm Hg or less, and 357 ml in those patients with atrial pressures above this level (see Figure 7). The difference between these means is statistically significant, the p value being less than 0.02. If those cases with calculated pulmonary vascular resistance above 500 dynes-sec-cm^{-5} are excluded, the effect of elevated left atrial pressure upon pulmonary blood volume is even more evident, the means of these two groups being 264 and 403 ml per m^2 ($p = < 0.01$).

A markedly elevated pulmonary vascular resistance is apparently associated with smaller volumes than would be expected from the pressure-volume relationship seen in the low resistance group. This is not a surprising finding if it is assumed that an elevated pulmonary vascular resistance represents vasoconstriction or mechanical obstruction of the pulmonary vessels. These processes may involve the pulmonary veins as well as arteries. Simon has demonstrated radiologic evidence of regression of engorgement of the pulmonary venous system in a group of cases with severe elevations of left atrial pressures as compared to a group with only moderate left atrial hypertension (28).

Figure 6 shows a similar relationship between the pulmonary arterial pressure and pulmonary blood volume. The association is more evident in the absence of pulmonary vascular disease, when mean pulmonary arterial pressure changes merely reflect rises of left atrial pressures. With the onset of severe elevation of pulmonary vascular resistance, these three parameters no longer exhibit the same dependence upon one another.

As has been pointed out by Burton (29), the factor which determines the volume of a vessel is the transmural pressure, or the difference in pressures within the lumen and exterior of the vessel. This transmural pressure does not necessarily bear a direct relationship to the gradient across a vascular bed from arterial to venous segment. Thus, it is understandable that a correlation was not observed between the pulmonary blood volume and the pressure difference across the pulmonary circulation (pulmonary arterial mean − left atrial mean pressure) or the pulmonary vascular resistance.

In view of the varying severity of the disease process among the diagnostic groups, it was not unexpected that the type of lesion, age, sex, or size of the patient had no apparent influence upon the volume of the pulmonary vascular compartment.

The studies of Lammerant (15) indicate a significant reduction in pulmonary blood volume during exercise and following food intake. Redistribution of blood with the upright position will result in a shift of blood out of the thorax and to the lower extremities. These influences, however, were not in effect in the patients of this study, who were all fasting and in the prone position.

It is apparent that there is no single determining factor governing the pulmonary blood volume in disease states, and it is concluded that a number of opposing influences are in operation whose combined effect is to maintain this volume of blood in the lungs within a relatively narrow range. While it is probable that increased pulmonary venous pressure, by producing distention of that vascular segment and also of the arterial compartment, tends to cause an increase of pulmonary blood volume, at the same time pulmonary vasoconstriction or vascular obliteration may be present and exert an opposite effect. Under such conditions the pulmonary blood volume as a whole might show no dramatic change.

SUMMARY

A method for the measurement of pulmonary blood volume in man has been described, utilizing the product of the cardiac output and the pulmonary mean transit time as determined by simultaneous indicator injections into pulmonary artery and left atrium.

The method has been applied to the study of 45 patients during combined right and left heart catheterization. The assumptions on which this

method is based are discussed, possible sources of error are considered, and the results are compared with previous studies of the pulmonary blood volume. The values obtained are essentially the same as those reported by Kunieda and Milnor and associates. They are significantly smaller than those previously reported by others, and are thought to approach more closely the true volume of the pulmonary vascular compartment.

In the absence of severe pulmonary vascular disease, there appears to be a direct relationship between the volume of blood in the lungs and the pressure within the pulmonary venous system. However, in the presence of vasoconstriction or mechanical obstruction of the pulmonary vessels, as reflected by an elevated vascular resistance, this relationship is altered and further elevations in pressure are not accompanied by volume increases of the same magnitude.

It is evident that a number of interrelated and opposing forces determine the volume of blood in the lungs, and their combined effect is to maintain this volume within a relatively narrow range.

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A METHOD FOR THE DETERMINATION OF THE VOLUME OF AIR IN THE LUNGS: MEASUREMENTS IN CHRONIC PULMONARY EMPHYSEMA *

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The present study is concerned with the measurement of the functional residual capacity (FRC) in chronic pulmonary emphysema by a gas dilution method, i.e., the washout of nitrogen from the lung during prolonged oxygen breathing.

There have been many methods in use to measure the FRC (or the residual volume) since Davy in 1800 measured his own residual volume by rebreathing hydrogen, which he had made himself, from a balloon (1). These methods, both the open circuit (washout) and closed circuit (rebreathing from a balloon or spirometer), have in general followed a consistent historic trend in that longer and longer periods of washout or rebreathing have been required in later methods: Davy in 1800 rebreathed for 20 seconds (1), Gréhant in 1864 for 6 inspirations (2), Van Slyke and Binger in 1923 for 5 minutes (3), Christie and Bates for 7 minutes (4, 5), Darling, Cournand and associates for 7 or 11 minutes (6, 7) and Briscoe, Becklake and Rose for 12 minutes (8, 9). In justice to these earlier workers it should be mentioned that most of those quoted above and others (10, 11) realized that to get the best results in emphysema somewhat longer studies were desirable.

More recent studies of the N_2 or helium washout in patients with chronic pulmonary emphysema have found detectable quantities of these inert gases being washed out of the lungs, distinct from those washed out from tissues, even after 20 to 40 minutes of O_2 breathing (12–18).

The present study describes a method in which all the N_2 eliminated from the lung during 15 minutes, or longer, of oxygen breathing is measured. Moreover, the quantity of N_2 which would be eliminated in a longer study is estimated by integrating the exponential washout function in the least ventilated alveoli. The advantage of this procedure lies in eliminating the need for an alveolar sample to estimate the N_2 remaining in the lung at the end of the study. Such samples are misleadingly low in N_2 in chronic pulmonary emphysema and result in underestimation of the FRC.

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METHODS

Functional residual capacities were measured by the conventional method of Cournand, Baldwin, Darling and Richards (7). They were also measured at a different time by a new method to be described here. This method is a development both of that used by Blair, Hickam and Frayzer with helium (14, 15) and by Briscoe and associates with N_2 (19). It utilizes a system of two balloons (Figure 1) which collect the gas expired in alternate 30-second periods. At the end of each 30-second collection, the balloon is emptied into the Tissot spirometer by pressing it between two boards suspended on either side of it. The mixed expired gas expelled from each balloon in turn is analyzed with a N_2 meter. Thus, every 30 seconds the volume expired and the mean concentration of N_2 in that volume are known. The study ends after 15 to 25 minutes.

I. Nitrogen washout curve. Figure 2 shows the data in one study in which there were 32 balloon collections during 16 minutes of O_2 breathing. In each collection the volume measured on the Tissot spirometer and the difference between the inspired N_2 concentration and that of the mixed expired gas in the balloon were measured. Their product yields the quantity of N_2 eliminated from the body in that half minute. By subtracting the quantity of N_2 eliminated from the tissues ($\dot{V}'_{N_2}$) in each period, the quantity of N_2 eliminated from the lungs ($\dot{V}_{N_2}$) in each period was determined and plotted on semilogarithmic paper.

At the beginning of O_2 breathing, $\dot{V}_{N_2}$ is momentarily about 81 per cent of expired gas volume ($\dot{V}_E$). In the first 5 minutes it falls steeply due to the rapid washout of N_2 from the well ventilated groups of alveoli. After about 5 minutes $\dot{V}_{N_2}$ is still considerable and only slowly decreasing. This is due to the extremely small ventilation of a large group of alveoli whose volume and ventilation can be estimated from a straight line drawn through this slow phase. The major part of the total N_2 initially present in

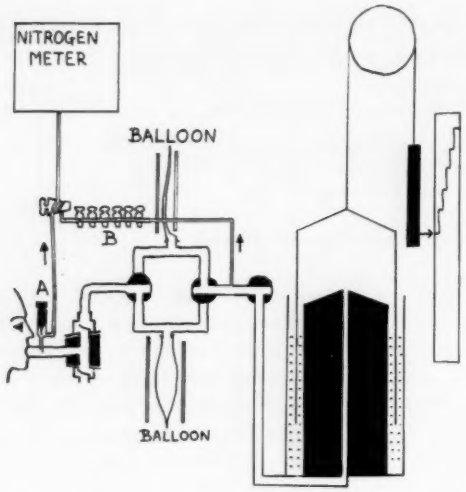

FIG. 1. SCHEMATIC REPRESENTATION OF THE EQUIPMENT USED IN THE PRESENT STUDY. The needle valve (A) samples at the mouth, and the long line, with glass stopcock leak (B), samples mixed expired air; the balloon stopcock assembly is made from anesthesia balloons and standard plumbing fittings. The valve box at the mouth is part of a 5-way stopcock (Respiration Aids, 255 E. 148 St., New York 51, N. Y.) here shown connected for oxygen breathing. When the mouthpiece is connected to air as the inspirate, the expirate goes to waste; these two channels used during air breathing are not shown here.

the lungs was determined without assumptions by adding the 32 measurements obtained in 16 minutes $\left(\int_0^{16} \dot{V}_{N_2} \cdot dt\right)$. The smaller quantity which would have been washed out thereafter was estimated by extrapolating the slow phase and integrating to determine $\int_{16}^{\infty} \dot{V}_{N_2} \cdot dt$ under the straight line in Figure 2.

II. Details of equipment and procedure. The dead spaces of the two anesthesia balloons used were 250 and 330 ml, these being the volumes of the tubes between the mouth and the balloons. To correct for this dead space the midpoint of each collection should be taken to be about 4 seconds earlier than the middle of the 30-second period. This has a negligible effect in studies on patients with chronic pulmonary emphysema. Both balloons were flushed with medical oxygen before each study, until the N_2 meter showed that the gas leaving the balloon contained no more than 0.025 per cent more N_2 than the medical oxygen, this figure being the limit of resolution of our N_2 meter.

The stopcocks controlling admission and displacement of gases alternately to and from the balloons were mechanically linked, so that when either balloon was connected to the Tissot spirometer, the other was automatically connected to the mouth. These stopcocks and their associated T and L pieces are standard $\frac{3}{4}$ inch plumbing supplies.

Inspired and expired gas were sampled for about 15 seconds in each half-minute period through a needle valve at the mouth. The conventional needle valve type of leak into the vacuum system of the N_2 meter has the advantage of quick response, but it is unreliable for accurate analysis since it is sensitive to changes in hydration and temperature, and to blockage by air-suspended particles. For the analysis of mixed expired gas in the balloons, 6 glass stopcocks connected in series gave a more reliable leak with a line delay of 3 seconds and a 90 per cent response time of half a second, when adjusted to produce a pressure in the vacuum system of 2 mm.

The N_2 meter was calibrated before and at the end of each study with known gas mixtures (air, medical O_2, and 2.2, 8.6, 16.6 and 42 per cent N_2 in O_2).

Sources of nitrogen other than the lungs were controlled as follows. None of the subjects had perforated ear drums. They were instructed not to belch or to allow leaks at the mouthpiece and their mouths were watched continuously by a second observer. Between balloon analyses the N_2 meter was connected to sample inspired and expired gas at the mouth for about 15 seconds in every half-minute period. The record of N_2 concentration at the mouth obtained via the needle valve showed no leaks.

III. Symbols. It is useful here to comment on the symbols used in this report. They are the same as those used previously in this laboratory (18–20), except in these respects: $\dot{V}'_{N_2}$ is the rate of nitrogen elimination from tissues, and $\dot{V}_{N_2}$ is the rate of nitrogen elimination from

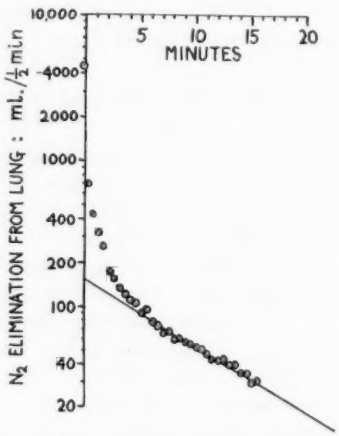

FIG. 2. DATA IN ONE STUDY ON A PATIENT WITH CHRONIC PULMONARY EMPHYSEMA. The abscissa shows the duration of O_2 breathing in minutes. The ordinate shows milliliters of N_2 at BTPS eliminated from the lungs in each half minute collection plotted on a logarithmic scale and corrected for tissue N_2. The line represents the extrapolated washout from the lungs after 16 minutes. The point before zero time represents the quantity of N_2 expired per half minute while the subject is breathing air.

TABLE I

Quantities of N_2 eliminated by a 70 kg man, in milliliters of N_2 BTPS during periods ending at the times indicated*

Time	Quantity/ 30 sec	Time	Quantity/ 60 sec
min	*ml N_2*	*min*	*ml N_2*
0.5	36	8	22
1.0	24	9	21
1.5	22	10	20
2	21	11	19
2.5	19	12	19
3	18	13	18
3.5	17	14	17
4	16	15	17
4.5	15	16	16
5	13	17	15
5.5	13	18	15
6	12	19	15
6.5	12	20	14
7	12	21	14

* These conditions are applicable to calculations of FRC. Volume (BTPS) = volume (STPD) × 1.21.

lungs corrected for elimination from tissues, i.e., $\dot{V}_{N_2} = \dot{V}_E(F_E - F_I) - \dot{V}'_{N_2}$. Whenever the symbol F is used for a gas concentration the chemical symbol is omitted, it being understood that we always refer to N_2 concentrations. A superscript refers to the time in minutes at which a rate of elimination or a concentration applies. Subscripts 1, 2 and 3 apply as before to phases or groups of alveoli with decreasing ventilation. M is the number of minutes in which a group of alveoli washes out 90 per cent of its N_2. $\dot{V}'$ is the flow rate from a group of alveoli at one instant during expiration and $\dot{V}$ is ventilation per minute, integrated over many breaths. F_A' is a gas concentration at the end of a forced expiration and $F\bar{A}$ is mean alveolar gas concentration, weighted by the volume of the differently ventilated alveoli.

IV. Tissue N_2 elimination. Thirty-minute washout studies on 5 normal subjects confirmed the rates of tissue N_2 elimination found by Robertson, Siri and Jones (12) and validated the use of our N_2 meter at low N_2 concentrations. Table I shows the rates of N_2 elimination used in calculating our data, which apply for a 70 kg man, and were therefore adjusted in proportion to the weight of each subject. These rates agree with the figures for N_2 elimination in 7 minutes already in use in this laboratory (21, 22).

V. Check on the method. In determining, by the use of balloons, the quantity of N_2 eliminated from the lungs over any given period, it is necessary to add the quantities in many balloon collections. The sum of so many quantities, each being a product of a volume and a concentration read from a calibration curve, might have a large error were there a small systematic error in each collection. Therefore, in 3 patients, as well as in a group of normal subjects not described here, the volume of lung N_2 eliminated both between 0 and 7 and between 7 and 15 minutes of O_2 breathing was simultaneously measured in three different ways as a check on the method: *1)* by summating the quantities of N_2 measured every half minute in the balloon collections by N_2 meter analysis and Tissot volume record between 0 and 7 and between 7 and 15 minutes; *2)* by collecting expired gases getween 0 and 7 and 7 and 15 minutes in two different Tissot spirometers and analyzing the mixed gas in each spirometer by means of the N_2 meter; and *3)* by analyzing gas samples from the same two spirometers with the Van Slyke apparatus.

Thus, in each subject there were two gas collections in which the quantity of N_2 eliminated from the lungs in 7 minutes was measured in three different ways. These three different methods of measurement agreed: the standard deviation of the various methods from the mean for all three was 99 ml N_2 BTPS in the 0 to 7 minute collection when the total quantity of N_2 averaged 3,479 ml, and 87 ml N_2 in the 7 to 15 minute period when the total quantity averaged 1,035 ml.

An additional check on the method is afforded by measurement of the quantities of N_2 eliminated in 7 minutes at different times by methods 1 and 3 in all 11 cases. Here, too, as is apparent from Table III, there is agreement within 10 per cent.

RESULTS

All 11 patients were disabled by severe chronic pulmonary emphysema with or without a bronchitic element. Their physical characteristics and maximal breathing capacity (MBC) are given in Table II. The results in this group will be presented under two main headings: *1)* the quantities of N_2 eliminated, as measured by different methods at various times during O_2 breathing, and the estimates of the FRC derived therefrom; *2)* the relative volume and ventilation of the least ventilated alveoli and their gas composition after 7 minutes of O_2 breathing.

I. Quantities of N_2 eliminated from lungs and FRC measurement. The FRC is the quantity of N_2 initially present in the lungs at the end of a resting expiration divided by 81 per cent, a

TABLE II

Physical characteristics and maximal breathing capacity (MBC) in 11 patients with chronic pulmonary emphysema

Subject	Age	BSA	MBC
	yrs	*m^2*	*L/min*
W.D.	61	1.64	16
M.L.	40	1.35	15
W.H.	58	1.50	20
A.Mc.	54	1.48	18
O.H.	66	1.85	18
S.G.	55	1.50	19
J.J.	67	1.57	14
L.L.	52	1.59	19
C.Gy.	56	1.46	16
C.Gi.	60	1.77	46
T.L.	58	1.53	19
Mean	57	1.57	20

TABLE III

Quantities of N_2 eliminated and the FRC: Comparison between new method and conventional method in 11 cases of chronic pulmonary emphysema

Subject	Quantities of N_2 eliminated* 0–7 min	7–15 min	15–∞ min	0–∞ min	FRC	Differences between two estimates of FRC	
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	%
W.D.	3,623 [3,823]	1,324	669	5,616	6,933 [5,245]	1,688	32
M.L.	2,335 [2,125]	686	383	3,404	4,202 [2,847]	1,355	48
W.K.	3,752 [3,725]	932	225	4,909	6,060 [5,094]	966	19
A.Mc.	2,517 [2,313]	433	173	3,123	3,856 [3,052]	804	26
O.H.	4,190 [4,215]	1,224	816	6,230	7,691 [6,222]	1,469	24
S.G.	2,687 [2,485]	663	350	3,700	4,567 [3,396]	1,171	34
J.J.	3,449 [3,461]	1,089	326	4,864	6,005 [4,873]	1,132	23
L.L.	2,740 [2,610]	826	537	4,103	5,065 [3,505]	1,560	45
C.Gy.	4,429 [3,995]	948	612	5,989	7,393 [5,368]	2,025	38
C.Gi.	2,497 [2,637]	999	533	4,029	4,974 [3,600]	1,374	38
T.L.	2,903 [2,985]	836	545	4,284	5,289 [4,053]	1,236	30
Mean	3,192 [3,125]	905	470	4,568	5,639 [4,296]	1,344	32

* All quantities of N_2 are at BTPS and corrected for N_2 from tissues. Figures in brackets are those obtained by the conventional method.

figure considered to correspond to the initial concentration of N_2 in the lungs. The quantity initially present theoretically corresponds to the quantity collected during the washout with O_2. This quantity, and hence the FRC, were determined by the conventional and by the new method. These determinations are summarized in Table III.

The first four columns of this table give the respective quantities of N_2 eliminated from the lungs: from 0 to 7 minutes, $\int_0^7 V_{N_2} \cdot dt$; from 7 to 15 minutes, $\int_7^{15} \dot{V}_{N_2} \cdot dt$; from 15 to ∞ minutes, $\int_{15}^{\infty} V_{N_2} \cdot dt$; and from 0 to ∞ minutes, the total amount of N_2 eliminated from the lungs if the study had continued indefinitely. These figures are corrected for the N_2 elimination from tissues and are based on the half-minute balloon collections. The lower figures in the first column are the quantities of N_2 eliminated from lungs as measured in the Tissot spirometer by the conventional method. Although measured on a different occasion, these data agree well with the results of the balloon collection. The data in column 3 are derived from the plotted N_2 washout curve by algebraic integration of the extrapolation of the slow phase from the end of the study to infinity:

$$\int_{15}^{\infty} V_{N_2} \cdot dt = {}^{15}V_{N_2} \cdot M_3/2.3$$

where M_3 is the time taken for the slow phase to fall through one logarithmic cycle, 2.3 is $\log_e 10$, and ${}^{15}\dot{V}_{N_2}$ is the rate of N_2 elimination from the lungs at 15 minutes.

It can be seen from the mean values that in patients with severe emphysema only 70 per cent of the lung's N_2 is eliminated in the first 7 min-

utes of O_2 breathing, 20 per cent between 7 and 15 minutes and the remaining 10 per cent thereafter.

The FRC determined by the quantity of N_2 eliminated during prolonged washout is $\int_0^\infty \dot{V}_{N_2} \cdot dt/0.81$. It has an average value of 5,639 ml. The FRC determined by the conventional method is $\left(\int_0^7 \dot{V}_{N_2} \cdot dt\right) \div (0.81 - {}^7F_A')$ and has an average value of 4,296 ml. The new method gives an FRC which ranges between 0.804 and 2.025 L higher than the conventional method, with a mean difference of 1.344 L; the percentage difference ranges between 19 and 48 per cent, with a mean difference of 32 per cent.

In Table IV are given some other lung volumes which are derived from the FRC. Values based on the conventional method are listed between parentheses. Significantly, the residual volume/total lung capacity (RV/TLC) ratio is relatively little affected by these large changes in both volumes (1.4 L), its average value increasing from 62 per cent with the conventional method to 70 per cent with the new method.

TABLE IV

Residual volume (RV) and total lung capacity (TLC)

Subject	RV*	TLC*	RV/TLC*
	L	*L*	%
W.D.	6.2 [4.4]	8.6 [6.8]	72 [64]
M.L.	3.1 [1.7]	5.2 [3.8]	59 [45]
A.Mc.	3.5 [2.6]	5.2 [4.3]	67 [61]
O.H.	6.9 [5.4]	9.0 [7.5]	77 [72]
J.J.	5.4 [4.2]	7.0 [5.9]	77 [72]
L.L.	3.9 [2.8]	5.7 [4.6]	69 [62]
C.Gy.	6.7 [4.7]	8.4 [6.3]	80 [73]
C.Gi.	4.3 [2.9]	7.9 [6.4]	63 [44]
T.L.	4.2 [2.9]	6.6 [5.4]	62 [54]
Mean	4.9 [3.5]	7.0 [5.7]	70 [62]

* The upper figures are derived from the FRC determined by the method described in this paper. The figures in brackets are based on the conventional method.

TABLE V

Relative volume (L_3/L_T) and ventilation ($\dot{V}_{A_3}/\dot{V}_E$) of the least ventilated alveoli, and alveolar nitrogen concentrations

Subject	L_3/L_T	$\dot{V}_{A_3}/\dot{V}_E$	Alveolar N_2 concentrations*		
			F_{A_3}†	$F\bar{A}$†	F_A'†
	%	%	%	%	%
W.D.	84	11	32	28	8.1
M.L.	67	6	39	26	6.3
W.H.	68	9	23	16	7.8
A.Mc.	64	6	24	15	5.2
O.H.	74	6	36	27	13.2
S.G.	74	7	30	22	7.8
J.J.	74	10	29	22	9.9
L.L.	76	5	36	27	6.5
C.Gy.	58	5	37	21	6.6
C.Gi.	76	3	40	30	7.7
T.L.	67	3	37	24	7.4
Mean	71	6	33	23	7.9

* After 7 min of O_2 breathing.

† F_{A_3}, alveolar N_2 concentration in least ventilated alveoli; $F\bar{A}$, mean alveolar N_2 concentration in all alveoli; F_A', alveolar N_2 concentration estimated by forced expiration.

II. Characteristics of the least ventilated alveoli and their effects upon alveolar gas composition after 7 minutes of O_2 breathing. Table V gives two parameters determined by the slow phase of the washout curve (19): L_3/L_T is the fraction of the FRC which is very poorly ventilated and $\dot{V}_{A_3}/\dot{V}_E$ is the fraction of expired ventilation which comes from these spaces. On the average, 71 per cent of the FRC was ventilated by 6 per cent of the minute ventilation. These characteristics have a profound effect upon the significance and validity of alveolar gas sampled after 7 minutes of O_2 breathing.

The concentration of N_2 in the least ventilated alveoli after 7 minutes of O_2 breathing (${}^7F_{A_3}$) is estimated from the washout rate of the slow phase:

$$ {}^7F_{A_3} = 0.81 \times e^{-(7 \times 2.3)/M_3}. $$

It ranges from 23 to 40 per cent with a mean of 33 per cent. The true mean alveolar N_2 concentration (${}^7F\bar{A}$) at this time, when the more ventilated alveoli contain negligible concentrations, is ${}^7F_{A_3} \times L_3/L_T$. This ranges from 15 to 30 per cent with a mean of 23 per cent. The forced mixed alveolar sample (F_A') at this time ranges from 5.2 to 13.2 per cent with a mean of 7.9 per cent.

DISCUSSION

I. The magnitude of the discrepancy. There is a large discrepancy, up to 2.0 L, in the FRC measured by the two methods in patients with emphysema. It is worthwhile to establish this firmly, since some earlier studies found that the N_2 washout curve yielded essentially the same FRC, even in emphysema, as did the conventional method (13). This discrepancy is not due to differences in measurements of quantities of N_2 eliminated from the lungs as shown by Table III. Furthermore, measurements in normal subjects, not described here, showed good agreement between the two methods. This discrepancy is largely due to the fact that the conventional method uses a forced expiration to estimate the average concentration of N_2 remaining in the lung after 7 minutes of O_2 breathing. Moreover, it has been shown that the least ventilated alveoli, which constitute on the average 71 per cent of the FRC, are ventilated by only 6 per cent of the minute ventilation (Table V). In order that the forced alveolar sample be representative for the measurement of the FRC, it is necessary that these alveoli contribute 71 per cent of the gas in this sample. If they did so, this sample ($^7F\bar{A}$) would, on the average, contain 23 per cent N_2. The forced alveolar sample ($^7F_A'$), with a mean composition of 7.9 per cent N_2, therefore grossly underestimates the mean N_2 concentration in the lungs after 7 minutes of O_2 breathing.

Studies of the alveolar N_2 concentration after a single breath of O_2 have clearly shown that the least ventilated alveoli contribute more to the expirate at the end of the forced expiration (23, 24). From such data it is not possible to determine how much more they contribute, since the N_2 concentration within the differently ventilated alveoli is not measured. The N_2 washout method permits an estimate of the N_2 concentration in the least ventilated alveoli after 7 minutes of O_2 breathing. Hence, we can determine how much these alveoli contribute to the sample taken at the end of a forced expiration.

$$F_A' = (\dot{V}'_3F_{A_3} + \dot{V}''_2F_{A_2} + \dot{V}'_1F_{A_1})/(\dot{V}'_1 + \dot{V}'_2 + \dot{V}'_3)$$

where $\dot{V}'$ refers to an instantaneous flow rate at the end of the expiration, and the subscripts refer to groups of alveoli. Since F_{A_2} and F_{A_1} are practically zero at 7 minutes of O_2 breathing

$$F_A'/F_{A_3} = V'_3/(\dot{V}'_1 + \dot{V}'_2 + \dot{V}'_3).$$

Using our mean values, this ratio is $7.9/33 = 24$ per cent. In other words, the poorly ventilated alveoli, which in this study constitute 71 per cent of the FRC, contribute only 24 per cent of the gas expired at the end of a forced expiration.

While this is four times as much as the 6 per cent which these alveoli contribute to mixed expired gas, it is not enough to make the forced expiratory sample a valid one. The better ventilated alveoli, which constitute only 29 per cent of the lung volume, still contribute 76 per cent of the gas expired, even at the end of a forced expiration. The use of such an alveolar sample to estimate the quantity of N_2 remaining in an unevenly ventilated lung must lead to an underestimate of the FRC. The only question is as to the degree of underestimation. This, in severe chronic pulmonary emphysema, is as indicated in Table III.

II. Validity of high FRC's in the literature. Determination of the FRC by mixing an inert gas into a spirometer of decreasing volume (due to oxygen consumption) tended to overestimate the FRC in patients with chronic pulmonary emphysema (4). This was due to the "N_2 lag" phenomenon (25, 26). However, from time to time, very high estimates of the FRC by valid methods in the hands of careful workers are found in patients with chronic pulmonary emphysema, and published. This applies both to the closed (27) and the open circuit methods (12, 14, 15, 18, 19) and especially when the studies are prolonged. In the past, values over 6 L have sometimes been presented apologetically by their authors and subject to a certain amount of skepticism. They have, for example, been attributed to leaks at the mouth or in the equipment used, to belching of abdominal gas, or to perforated ear drums. It was believed that the FRC in emphysema could not exceed the TLC of a normal lung (28). It now seems that some of these very large FRC's may, in fact, be acceptable measurements.

III. The concept of trapped gas. DuBois, Bedell and associates (29, 30), in comparing the thoracic gas volume measured by a body plethys-

mographic method (31) with the FRC measured by the conventional method, found that the latter was less by an amount which varied between 0.69 and 2.38 L in emphysematous subjects. At that time they attributed their higher values by the plethysmographic method to trapped gas. They conceived that there were alveoli which were closed and which therefore did not wash out N_2, although they did, like freely communicating alveoli, affect the compressibility of the lung by the chest wall when the shutter at the mouth was closed. It now seems that if great care is taken to collect and measure all the N_2 washed out of the alveoli in prolonged O_2 breathing, a discrepancy of the same order of magnitude is observed. In the present study, the new method gave values which exceeded those of the conventional method by between 0.828 and 2.082 L. This suggests that the present method is measuring the same lung spaces as did the plethysmographic method.

That these spaces are included in the FRC, as measured in the present study, indicates that their gas is not completely trapped. Any gas-filled space in the body must be replenished from time to time if it is not to collapse; the spaces we measure must also contribute to expired gas, not necessarily at every breath but at some times during 15 minutes. In some patients with large cysts in otherwise normal lungs, we have observed that there are variations in the N_2 washout curve, suggesting that some pulmonary spaces empty intermittently at intervals of several minutes. This has been observed by others also (32). We regard these spaces as ventilated, not as reservoirs of trapped gas, even though they do not contribute to expired gas at every breath.

IV. The time needed between studies. The quantity of N_2 eliminated in successive conventional FRC determinations in this laboratory indicates that in chronic pulmonary emphysema the second study showed a smaller quantity of N_2 eliminated. This review applied to paired studies in each of 15 consecutive cases with emphysema both in 1940 and in 1958. The mean differences were 8.6 per cent of the total quantity of N_2 in 1940, and 9.0 per cent in 1958. A waiting period of half an hour between studies was usually allowed. This seems to be inadequate in emphysema, and a waiting period of at least 1 hour between duplicate determinations of FRC is now being used with the new open circuit method.

V. Relative merits of various methods used for the measurement of FRC. The open circuit method described here has the advantage of giving a more accurate estimate of the volume of the lungs in emphysema than the conventional method, which is in error by as much as 48 per cent in this series. It should be pointed out that when Cournand and co-workers (7) developed the conventional method, they were very much aware of the possibility of such an error in patients with emphysema. They measured the discrepancy between wash-in and wash-out FRC determinations, which is due to poor distribution and inadequate representation by the terminal forced expiration sample. In their subject, M.K., there was a discrepancy of 865 ml at 7 minutes, which fell to 590 ml when the study was continued for 11 minutes (6). This is in the lower part of the range of the discrepancies discussed here.

The mean values in Table II show that in a typical case of severe chronic pulmonary emphysema about 70 per cent of the lung's nitrogen has been washed out in 7 minutes and about 90 per cent is washed out in 15 minutes. If the 7-minute alveolar sample were in fact representative, it should indicate that 30 per cent of the nitrogen initially present still remains in the lungs, i.e., it would have a value of about 24 per cent in a typical case. In fact, this is more than the highest values which are actually observed. Alveolar samples at the end of a 7-minute washout can lead to large errors; but if a 15-minute collection were made, at which time a typical case has washed out 90 per cent of the nitrogen initially present, a low alveolar sample could cause an error of, at most, 10 per cent of the FRC. However, if 15-minute washouts were done with the conventional Tissot method of collecting the gas, it would be necessary to use more, or larger, collecting spirometers and to analyze for very low concentrations of N_2.

The method described here requires considerably more time, skill in handling of equipment, and calculation than the conventional method, and it may therefore be unsuitable for routine use. An alternative procedure is the body ple-

thysmographic method (31). This requires more instrumentation and more skill in maintenance and testing of equipment, but the duration of the study and the time spent in calculation is less. Good as this method is, however, it must underestimate, by about 30 per cent, the volume of lung spaces too large to allow rapid heat exchange between gas and tissue (33). To attain comparable accuracy in severe emphysema by the closed circuit helium dilution method, prolonged studies are necessary and the leakage or diffusion of helium from the circuit must be considered. It seems then that the measurement of the volume of air in the lungs of patients with severe chronic pulmonary emphysema presents an even greater challenge to our ingenuity than has been suspected heretofore. However, the method which at present seems the least open to objection is the one described here, which measures the total quantity of N_2 initially present in the variously ventilated parts of these lungs, and washed out of them during O_2 breathing.

SUMMARY

A method is described for collecting and measuring the quantity of N_2 washed out of the lungs every half minute during oxygen breathing. The total quantity of N_2 eliminated in 15 minutes of O_2 breathing was measured in 11 patients with chronic pulmonary emphysema. The quantity of N_2 which would have been eliminated in more prolonged studies was estimated by extrapolating the N_2 washout curve. The functional residual capacity (FRC) so determined exceeded that obtained by conventional FRC measurements by between 0.804 and 2.025 L. The magnitude of this discrepancy agrees well with that seen when body plethysmographic estimates are compared with conventional measurements of the FRC. It is suggested that this new method measures the volume of the same intrapulmonary spaces as does the body plethysmograph.

The composition of forced expiratory gas samples in chronic pulmonary emphysema is discussed.

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THE EFFECTS OF INTRAVENOUS INFUSIONS OF VALINE-5 ANGIOTENSIN II AND OTHER PRESSOR AGENTS ON URINARY ELECTROLYTES AND CORTICOSTEROIDS, INCLUDING ALDOSTERONE *

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Previous studies from this laboratory (1–4) have shown that: *1)* there is a statistically significant increase in the mean urinary aldosterone excretion from groups of patients with essential, renal or malignant hypertension, as compared with that of normal subjects similarly studied; *2)* 43 per cent of patients with essential hypertension have urinary aldosterone values above normal limits; *3)* there is an excessive fluctuation of daily urinary aldosterone in hypertensive patients, from normal to above normal levels, which is most prominent in the early phase of the disease; and *4)* mean urinary pregnanetriol is significantly decreased ($p < 0.001$) in the same groups of hypertensive patients, and the pregnanetriol/aldosterone ratio is below the lower limits of normal range in 92 per cent of all patients studied with arterial hypertension.

The findings of a mean increase in urinary aldosterone and of its excessive daily fluctuation in hypertensive patients have recently been confirmed by Romanelli, Biancalona and Matterazzi (5), Venning, Dyrenfurth, Dossetor and Beck (6) and Schwartz (7). However, Laragh and co-workers (8), using a method devised by Ulick, Laragh and Lieberman (9) for the measurement of aldosterone secretion rate, found normal values in 8 patients with benign essential hypertension and significantly increased secretion rates in 5 of 8 patients with advanced hypertension and in 14 of 15 patients with malignant hypertension. On the other hand, in a preliminary study, Muller found normal aldosterone secretion rates in patients with malignant hypertension (10).

It appears however, that this disturbance in adrenocortical regulation may not be the basic or the sole factor in the pathogenesis of hypertension. Both clinical and experimental evidence strongly suggest that the kidney plays a definite role. We therefore studied the possibility of a relationship between the renal pressor system and aldosterone.

Our preliminary results (11), showing a marked stimulatory effect of angiotensin II on urinary aldosterone, have been confirmed in the present study.

Seven normal subjects were studied. Continuous intravenous infusions of glucose, angiotensin, norepinephrine and phenylephrine were administered for periods of 7 to 14 hours, and urines were analyzed for sodium, potassium, aldosterone, cortisol, cortisone and their tetrahydro derivatives. The results obtained show a marked and specific tropic effect of angiotensin on aldosterone excretion.

SUBJECTS AND METHODS

The subjects were 7 healthy male volunteer medical students, maintained on a fixed sodium and potassium intake (102 and 90 mEq per day, respectively) for 5 days prior to and during the experimental period. They were admitted to hospital for metabolic balance for the duration of the experiments. Urine was collected every 24 hours, except on infusion days, when it was collected during the period of infusion and again separately for the rest of the 24 hour period (post-infusion period). Six infusions were carried out with synthetic valine-5 angiotensin II, two with norepinephrine [1] and two with phenylephrine.[2] The pressor substances were diluted in 5 per cent

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[1] Winthrop, Levophed.

[2] Winthrop, Neosynephrine.

TABLE I

Effects of i. v. valine-5 angiotensin II infusions in normal male volunteers *

Patients	Dose of angiotensin	Aldosterone† (μg/day)			
			Day 2		
		Day 1	Angiotensin infusion	Post-angiotensin infusion	Day 3
J.M.	2 mg/14 hrs	14	52	69	68
R.A.	3.1 mg/7 hrs	10	14	47	
	3.4 mg/13 hrs	11	105	98	28
F.M.	1.2 mg/8 hrs	19	32	42	23
J.-P. D.	0.9 mg/7 hrs	5	36	27	16
H.N.	0.9 mg/12 hrs	23	84	29	25

* On fixed Na and K intake (102 and 90 mEq/day, respectively).
† Nowaczynski, Koiw and Genest's procedure (21).

glucose in water and infused at a rate sufficient to maintain a constant elevation of diastolic pressure of at least 30 to 40 mm Hg above control levels, for periods ranging from 7 to 14 hours. Control infusions of 5 per cent glucose in water were given to 5 subjects.

Urinary sodium and potassium were determined with a model 52 C Perkin-Elmer flame photometer. The procedures used for hydrolysis, extraction, purification and determination of the steroids, as well as the technical details, have been previously described (3, 12, 13). These

TABLE II

Effects of i. v. infusions of angiotensin and norepinephrine in normal male subjects *

		Urinary excretion								
		K	Na	Aldosterone	Tetrahydroaldosterone	Cortisol	Cortisone	Tetrahydrocortisol	Tetrahydrocortisone zone†	Sum of "F"+"E" THF+THE
		mEq/day		*μg/day*		*μg/day*		*μg/day*		*mg/day*
R.A. (22 yrs)										
Day 1	Control	84	85	11	76	70	133	3,017	1,407	4.6
Day 2	Angiotensin inf. (3.4 mg/13 hrs)	78	31	105	505	218	227	2,382	6,792	9.6
	Post-inf. period	46	23	98	383	444	133	907	2,129	3.6
Day 3	Control	90	108	28	138	125	153	3,744	1,815	5.8
Day 4	Control	75	78	19	65	180	74	2,099	803	3.2
Day 5	Norepinephrine inf. (6.3 mg/7 hrs)	60	230	41	493	508	511	2,348	3,259	6.6
	Post-inf. period	65	26	26	73	106	246	990	1,445	2.8
J.M. (26 yrs)										
Day 1	Control	39	39	14	195	210	89	1,229	790	2.3
Day 2	Angiotensin inf. (2 mg/13.6 hrs)	81	14	52	336	248	97	1,916	1,357	3.6
	Post-inf. period	73	12	69	595	102	137	3,752	2,358	6.3
Day 3	Control	48	35	68	226	193	197	3,989	3,404	7.8
J.P.D. (21 yrs)										
Day 1	Control	67	58	5	71	107	140	1,024	1,768	3.0
Day 2	Angiotensin inf. (0.9 mg/7.3 hrs)	50	6	36	389	218	249	931	1,852	3.2
	Post-inf. period	69	31	27	249	93	85	699	966	1.8
Day 3	Control	95	39	16	56	229	197	1,954	1,162	3.5
H.N. (23 yrs)										
Day 1	Control	75	53	23	66	85	183	2,199	1,806	4.3
Day 2	Angiotensin inf. (0.9 mg/12.4 hrs)	70	16	84	113	137	311	876	3,955	5.3
	Post-inf. period	44	6	29	458	117	291	585	1,211	2.2
Day 3	Control	74	88	25	138	141	239	3,317	912	4.6

* On a fixed Na and K intake (102 and 90 mEq/day, respectively).
† The measurement of the "tetrahydrocortisone zone" by the blue tetrazolium reaction includes also that of tetrahydroaldosterone and of *allo*-tetrahydrocortisol.

procedures are essentially based on isolation of the various steroids in a high degree of purity. Identification of the individual steroids is based on chromatographic mobilities in two or three paper systems in comparison to mobilities of standard reference substances, on ultraviolet light absorption, reaction with blue tetrazolium (14) and isonicotinic acid hydrazine (15), absorption spectra in concentrated sulfuric acid (16–18) and in 100 per cent phosphoric acid (19–21).

Since the blue tetrazolium reaction was used for determination of tetrahydrocortisone, the indicated values include also those of the ring-A reduced metabolite of aldosterone, tetrahydroaldosterone (pregnane 3α-18,21-triol-11,20-dione) and of *allo*-tetrahydrocortisol (3α,11β, 17α,21-tetrahydroxy-*allo*-pregnane-20-one).

Mean recovery of added aldosterone to urine aliquots from normal subjects was 83 per cent. As we have emphasized in the past (21) the recovery is a direct function not only of the procedure itself, but mainly of the meticulousness in the technique of completely solubilizing residues, of their total transfer from beaker (or other container) to paper chromatograms and of complete elution of "steroids spots" from the chromatograms. Reproducibility in the hands of a well trained, experienced and meticulous technician is within 10 to 20 per cent, depending on the amount of steroid present in urine.

Peterson, Karrer and Guerra's modification of Silber and Porter's procedure was used for determination of urinary 17-hydroxycorticosteroids (22). Pregnanetriol was measured according to Bongiovanni and Eberlein's procedure (23). For the measurement of the ring-A reduced metabolite of aldosterone (24), the material eluted from the zone containing this compound in the ethylene glycol : toluene system was acetylated and chromatographed in the iso-octane, 1 : cyclohexane, 2 : ethylene glycol system (24). The reduced metabolite of aldosterone was measured as a triacetate using the blue tetrazolium reaction. Our assumption that the material measured is the ring-A reduced metabolite of aldosterone is based on the following facts: *1*) its similar chromatographic mobility to the metabolite A2 of aldosterone described by Sandor, Nowaczynski and Genest (24) in the following paper chromatographic systems—(*a*) for the free compound in toluene : ethylene glycol, and (*b*) for the acetate derivatives in toluene, 1 : iso-octane, 1 : 70 per cent methanol (Bush B 1 system) and iso-octane, 1 : cyclohexane, 2 : ethylene glycol; *2*) the same chromatographic mobility as the compound described by Ulick and Lieberman (25)

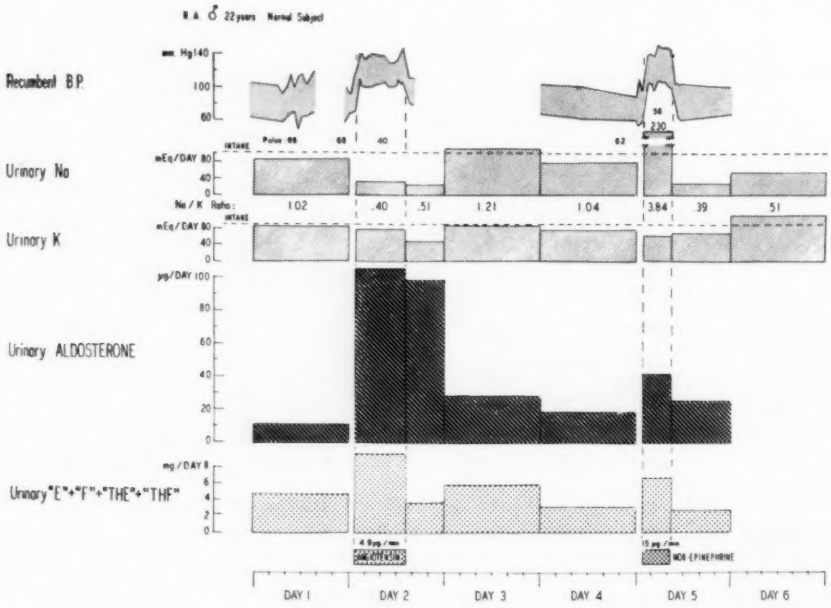

FIG. 1. EFFECT OF INFUSION OF ANGIOTENSIN (4.9 µG PER MINUTE FOR 13 HOURS) AND OF NOREPINEPHRINE (15 µG PER MINUTE FOR 7 HOURS) ON BLOOD PRESSURE, URINARY SODIUM, Na : K RATIO, POTASSIUM, ALDOSTERONE AND THE SUM OF SEPARATELY DETERMINED CORTISONE, CORTISOL AND THEIR TETRAHYDRO DERIVATIVES. Note the extreme stimulation of aldosterone which persisted for 10 hours after the infusion of angiotensin and which was accompanied by marked fall in urinary sodium and Na : K ratio and a slight rise in the 17-hydroxycorticosteroids. In contrast, the norepinephrine infusion produced a slight rise in urinary aldosterone coincident with a marked increase in urinary sodium and Na : K ratio.

in the benzene : 50 per cent methanol system; *3*) the identical spectrum in concentrated sulfuric acid to that given by metabolite A2 of aldosterone as described by Sandor and associates (24) and; *4*) the decreased polarity of the compound after acetylation, which indicates formation of a triacetate.

RESULTS

The over-all effect of the angiotensin infusions on urinary aldosterone of normal males is shown in Table I. The rate of angiotensin infused varied from 1.2 to 7.4 μg per minute. There is a two- to tenfold increase in urinary aldosterone during and/or immediately following the angiotensin infusions. This increase can persist for 1 day after the infusion. The mean urinary aldosterone excretion of the group was 12 μg per day during the control period, 54 during the period of infusion, and 52 during the post-infusion period.

The results of the daily excretion of sodium, potassium, and of cortisol, cortisone and their tetrahydro derivatives in four subjects in whom they were analyzed are shown in Table II. During the infusion of angiotensin, there is a very slight but consistent rise in the sum of these steroids above control levels. In two subjects, the sum of these steroids increased two- to threefold, but only once was the value definitely above the range of normal variations. The increase is mostly evident in the tetrahydrocortisone zone, which includes the tetrahydroaldosterone. The latter, when separated, accounted for part of the rise and showed changes parallel to those of aldosterone. The twofold increase in the sum of 17-hydroxycorticosteroids in Subject R.A. is still within the range of normal variations.

The infusion of angiotensin (3.4 mg per 13 hours) in Subject R.A. was accompanied and followed by a fall in urine volume, a sodium retention, and a tenfold increase in urinary aldosterone (Figure 1). Smaller increases in cortisol, cortisone and their tetrahydro derivatives were noted (Table II). The sum of the 17-hydroxycorticosteroids went up from 4.6 to 9.6 mg per day. The norepinephrine infusion (15 μg per minute for 7 hours) provoked a very marked natriuresis accompanied by an increase in aldosterone (twofold), tetrahydroaldosterone (eightfold), cortisol (threefold), cortisone (sevenfold) and "tetrahydrocortisone zone" (threefold). This occurred only during the period of the infusion.

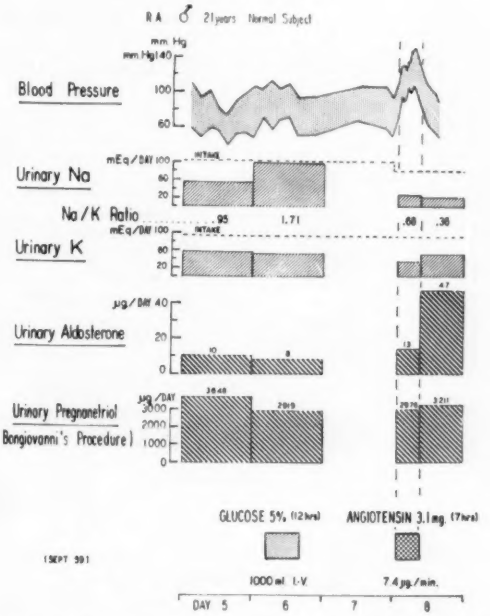

FIG. 2. EFFECTS OF INFUSIONS OF 5 PER CENT GLUCOSE IN WATER (1 L FOR 12 HOURS) AND OF ANGIOTENSIN (7.4 μG PER MINUTE FOR 7 HOURS) ON BLOOD PRESSURE, URINARY SODIUM, Na : K RATIO, POTASSIUM, ALDOSTERONE AND PREGNANETRIOL. Glucose infusion was accompanied by an increase in sodium output without any change in urinary aldosterone. In contrast, the angiotensin infusion was immediately followed by a fourfold rise in urinary aldosterone accompanied by a marked fall in sodium output and in the Na : K ratio.

Another experiment was performed on the same subject who received a control infusion of 5 per cent glucose for 12 hours and a second infusion of angiotensin (3.1 mg per 7 hours). No change in urinary aldosterone is observed during the glucose infusion, but the angiotensin administration is accompanied by a marked sodium retention and followed by a large increase in aldosterone (Figure 2).

In Subject J.M. (Figure 3), the infusion of angiotensin (2 mg per 14 hours) is accompanied and followed by sodium retention and a fivefold increase in urinary aldosterone excretion which persists through the day after infusion. The sum of the 17-hydroxycorticosteroids rose progressively from 2.3 to 7.8 mg per day.

In Table III are shown the results of two phenylephrine infusions on aldosterone excretion, which fell markedly during and immediately fol-

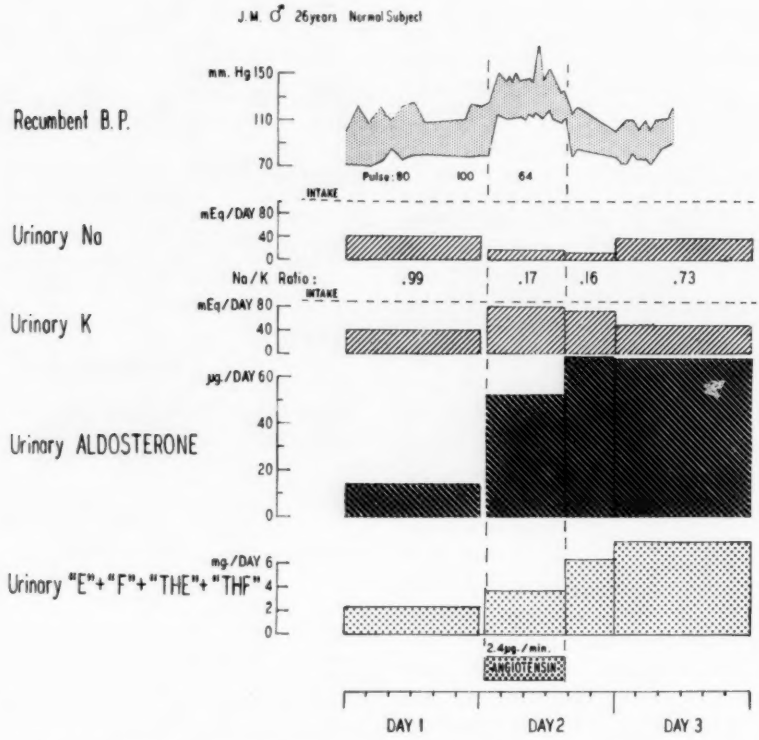

FIG. 3. EFFECTS OF ANGIOTENSIN INFUSION (2.4 µG PER MINUTE FOR 14 HOURS) ON BLOOD PRESSURE, URINARY SODIUM, Na : K RATIO, POTASSIUM, ALDOSTERONE AND THE SUM OF SEPARATELY DETERMINED CORTISONE, CORTISOL AND THEIR TETRAHYDRO DERIVATIVES. The very marked rise in urinary aldosterone during the infusion persisted immediately after the infusion and the following day and was accompanied by a marked fall in urinary sodium and Na : K ratio, and a significant increase in potassium. Urinary 17-hydroxycorticosteroids increased slightly and progressively.

lowing the infusions, while a pronounced natriuresis was produced.

Subject P.M., a hyper-reactor with blood pressure readings occasionally above 150/90 mm Hg, received an intravenous phenylephrine infusion at the rate of 10 µg per minute for 8 hours. Aldosterone fell significantly, while sodium excretion increased from a control level of 60 to 274 mEq per day (Figure 4). This subject did not show significant change in urinary aldosterone during a control 5 per cent glucose infusion.

Intravenous infusion of phenylephrine at the rate of 8.3 µg per minute for 8 hours in Subject G.T. (Figure 5) was accompanied by a fall in urinary aldosterone coinciding with a very marked natriuresis. This subject also received, 2 days before, a norepinephrine infusion, but unfortunately the urine aliquot collected during the infusion was accidentally lost before steroid determinations. There was no change in sodium

TABLE III

Effects of phenylephrine infusions in normal male volunteers *

Subjects	Urinary aldosterone†			
	Day 1	Day 2		Day 3
		Phenyleph. infusion	Post-phenyleph. infusion	
	µg/day	µg/day	µg/day	µg/day
G.T.	32	11	16	34
P.M.	26	12	17	12

* On fixed Na and K intake (102 and 90 mEq/day).
† Nowaczynski, Koiw and Genest's procedure (21).

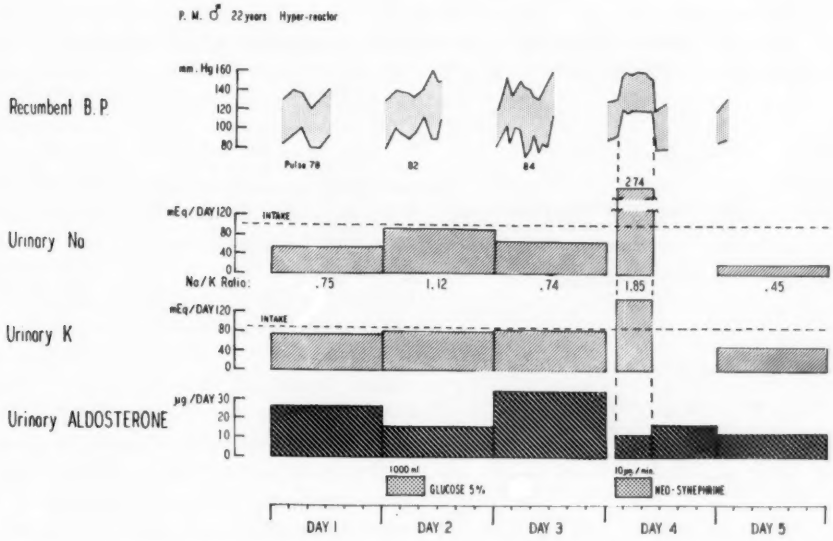

FIG. 4. EFFECTS OF INFUSIONS OF 5 PER CENT GLUCOSE IN WATER (1 L FOR 8 HOURS) AND OF PHENYLEPHRINE (10 μG PER MINUTE FOR 8 HOURS) ON BLOOD PRESSURE, URINARY SODIUM, Na : K RATIO, POTASSIUM AND ALDOSTERONE. In contrast to the effects of angiotensin, phenylephrine, infused at a rate sufficient to produce the same increase in diastolic pressure, provoked an important natriuresis accompanied by a marked rise in Na : K ratio and potassium, and by a significant fall in aldosterone.

excretion, and no increase in aldosterone was noted during the post-infusion period.

In another series of experiments previously done in our laboratory (*26*), we have studied the effects of 8-hour intravenous infusions of norepinephrine and of epinephrine in 5 per cent glucose on the urinary excretion of aldosterone and 17-hydroxycorticosteroids. Three normotensive sub-

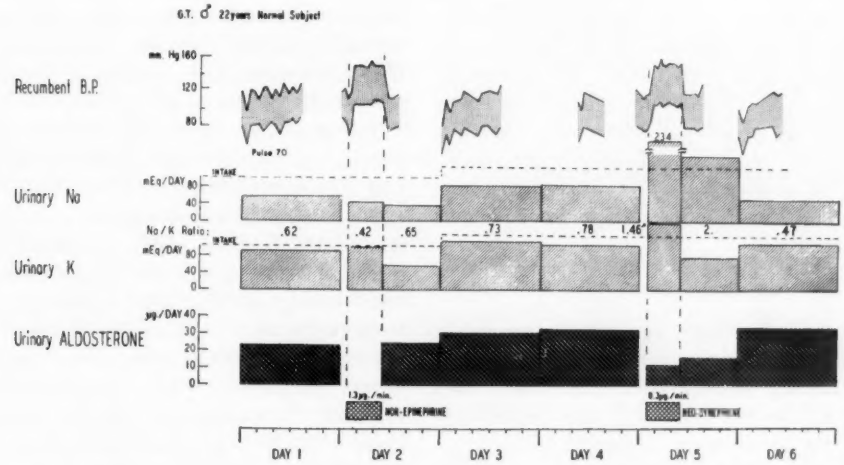

FIG. 5. EFFECTS OF INFUSION OF NOREPINEPHRINE (1.3 μG PER MINUTE FOR 8 HOURS) AND OF PHENYLEPHRINE (8.3 μG PER MINUTE FOR 8 HOURS) ON BLOOD PRESSURE, URINARY SODIUM, Na : K RATIO, POTASSIUM AND ALDOSTERONE. The patient's response to phenylephrine is exactly superimposable upon the one illustrated in the previous figure.

TABLE IV

Effects of 8-hour i. v. infusions of norepinephrine and epinephrine (in 5 per cent glucose)

Patients*	Age	Diagnosis	Aldosterone†			17-Hydroxycorticosteroids‡		
			Control glucose 5%	Norepin.	Epin.	Control glucose 5%	Norepin.	Epin.
			μg/day	μg/day	μg/day	mg/day	mg/day	mg/day
R.B.	25	Benign ess. hypert.	9	3	3	4.3	5.5	6.6
A.C.	18	Benign ess. hypert.	3	3	7			
W.C.	41	Benign ess. hypert.	8	8	9	6.3	8.0	7.9
C. de B.	18	Normal subject	7	8	11	4.2	5.6	5.2
T.P.	35	Schizophrenia	9	12	4			
A.G.	28	Obesity	5	5		1.6	5.5	

* Unrestricted diet.
† Nowaczynski, Koiw and Genest's procedure (21).
‡ Peterson and associates' modification of Silber-Porter (22).

jects and three patients with early benign essential hypertension were admitted to hospital and received unrestricted diets. On Day 1, a control infusion of 5 per cent glucose was administered for 8 hours. On Day 2, an 8-hour norepinephrine infusion in 5 per cent glucose was given at a rate sufficient to produce a slight increase in systolic (mean, 25; range, 12 to 41) and diastolic (mean, 15; range, 7 to 24 mm Hg) blood pressure, and an average decrease of 11 pulsations per minute (range, 5 to 16). On Day 3, an 8-hour epinephrine infusion in 5 per cent glucose was given at a rate sufficient to produce an average increase of 29 pulsations per minute (range, 19 to 40). Urines were collected only during the time of infusion and all results of urinary aldosterone and 17-hydroxycorticosteroids are expressed in micrograms and milligrams per day, respectively. The results illustrated in Table IV indicate that there is essentially no change in urinary aldosterone excretion. There is a very slight, but apparently not significant, rise in urinary total 17-hydroxycorticosteroids with equal distribution in both free and conjugated fractions.

DISCUSSION

In the six experiments performed, angiotensin markedly reduced urine volume, sodium excretion and the urinary sodium : potassium ratio. This confirms the results of Peart who consistently observed the same effect in normal subjects receiving angiotensin infusions (27–29). The effects of angiotensin administration in normal subjects appear to be twofold: *1*) a hemodynamic one with pronounced fall in glomerular filtration rate and filtered load of water, sodium, chloride and potassium with subsequent reduction in their excretion, an effect seen only when there is a significant rise in blood pressure; and *2*) a direct effect on the tubular reabsorption of water and sodium either per se or through an increase in aldosterone secretion, as the present experiments strongly suggest. Peart has demonstrated that the same effect on water and sodium excretion could be obtained without any change in glomerular filtration rate when angiotensin is infused at a very slow rate—insufficient to raise the blood pressure. It is also of great interest that, according to Peart's experiments, hypertensive patients show an increase in urine volume and natriuresis during angiotensin infusion. This effect, noted in 17 of 20 hypertensive patients, is completely opposite to that found in normal subjects (29).

The results we have obtained with angiotensin infusions confirm the experimental data of Tobian (30, 31) and of Hartroft, Pitcock and Hartroft (32, 33), who have demonstrated in rats a parallel relationship between width of adrenal zona glomerulosa secreting aldosterone and granularity of renal juxtaglomerular cells elaborating renin or renin precursor in response to changes in sodium intake. Our findings also agree with those of Deane and Masson (34), recently confirmed by Hartroft, Newmark and Pitcock (35) and by Gross (36, 37), who established that renin administration produces a rapid and marked increase in width of the adrenal zona glomerulosa in rats.

Another aspect of these studies is given empha-

sis by the recent experiments of Hilton (38). Using *in vivo* perfusion of adrenal glands in dogs, Hilton has demonstrated that vasopressin specifically stimulates cortisol secretion and does not influence that of aldosterone. Since vasopressin is closely related chemically to angiotensin, it is of interest to note that these two related polypeptides can specifically and differentially stimulate the secretion of the two main corticosteroid hormones.

If the studies of Laragh and co-workers (8) on the increased aldosterone secretion rate in human malignant hypertension are confirmed, despite the negative preliminary results of Muller (10), it is difficult to reconcile the urinary values found, for example, in patients with malignant hypertension, where 21 of 38 determinations in 26 patients were within normal range by our method (3, 21), and those of Laragh who found aldosterone secretion rates above 700 μg per day (normal range, 150 to 330 μg per day) in 11 out of 15 patients with malignant hypertension. This is somewhat surprising, since in the cases of primary aldosteronism in which high urinary aldosterone levels are almost always found, secretion rates below 650 μg per day have been found in 3 of 5 patients studied by the same authors. This would indicate quite important differences in metabolism of aldosterone in these two diseased states. The solution of these discrepancies must await further work, especially the serial measurement of secretion rate in the same patients as well as the simultaneous plasma level and urinary excretion of aldosterone. More study is also necessary to elucidate the factors responsible for the excessive fluctuation in urinary aldosterone of patients with benign hypertension.

The stimulatory effect of angiotensin on aldosterone appears quite marked and specific. Such effect was noted only once (a nearly twofold rise) in seven experiments with norepinephrine and not with phenylephrine or epinephrine. The increase in urinary aldosterone is probably not the result of the induced hypertensive state, since it is not encountered with phenylephrine and norepinephrine (with the one exception), although significant increases in blood pressure were achieved in all instances. Since some experimental data suggest that hyperfunction of the adrenal zona glomerulosa is accompanied by increased granularity of juxtaglomerular cells and renin content of kidneys, it would be of the utmost interest to determine plasma angiotensin levels in patients with proven primary hyperaldosteronism. Studies are actually under way in our laboratory to do simultaneous determinations of blood angiotensin (39), urinary aldosterone, pregnanetriol and pregnanetriol: aldosterone ratio in hypertensive patients.

SUMMARY AND CONCLUSION

Seven healthy young men were studied, under conditions of metabolic balance for sodium and potassium, for the effect of intravenous infusions of glucose, angiotensin, norepinephrine and phenylephrine on urinary electrolytes, aldosterone, cortisol, cortisone and their tetrahydro derivatives. Six angiotensin infusions done in five subjects were accompanied or followed by sodium retention, antidiuresis, a two- to tenfold increase in aldosterone excretion and a small and probably not significant increase in cortisol, cortisone and their tetrahydro derivatives. Two phenylephrine infusions stimulated diuresis and sodium excretion, and decreased aldosterone excretion. Urinary aldosterone did not increase during similar infusions of 5 per cent glucose in water, and in six out of seven infusions with norepinephrine.

These experiments establish that angiotensin has a marked and quite selective effect on aldosterone excretion in man. Its effect on urinary 17-hydroxycorticosteroids is very slight and probably not significant from the physiological point of view. This strongly suggests a direct evidence, in humans, of a definite correlation between the adrenal zona glomerulosa secreting aldosterone and the renal juxtaglomerular apparatus elaborating renin or renin precursor. It also opens new areas of research in the field of relationships between hormones of polypeptidic and steroidal nature.

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PULMONARY FUNCTION IN HYPERTHYROIDISM *

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Previous investigators, including Peabody and Wentworth in 1917 (1), have described dyspnea and a decreased vital capacity in hyperthyroid patients (2, 3). Following successful therapy of hyperthyroidism, an increase in the vital capacity to normal levels has been observed. The precise relationship of these changes to other measurable derangements of pulmonary or cardiac function has not been determined. Therefore, the present study was undertaken using recently developed techniques to determine the correlation between the changes in vital capacity in hyperthyroidism and changes in *1*) lung compliance, *2*) strength of the respiratory muscles, *3*) airway resistance, or other factors. Also, an attempt has been made to explain the symptom of dyspnea associated with hyperthyroidism (4, 5) in the absence of obvious congestive heart failure.

MATERIALS AND METHODS

Patients. The patients studied were from the wards or clinics of the Philadelphia General Hospital or the Hospital of the University of Pennsylvania. The diagnosis of hyperthyroidism was based upon *1*) clinical evaluation, *2*) I^{131} uptake by the thyroid gland, *3*) basal metabolic rate, and *4*) serum protein-bound iodine.

Thirteen patients, 12 of whom were female (Table I), were studied. The ages ranged from 21 to 55 years (mean age 41). In addition to other symptoms of hyperthyroidism, 9 patients had decreased exercise tolerance. Four described "hard breathing" or "inability to get enough air" on moderate exercise and 5 had shortness of breath on mild exertion. There was none with difficult breathing at rest.

One patient (RR) had acute pulmonary edema 1 month prior to study, treated with digitalization, low salt diet, and bed rest. Although she had not received antithyroid medication, there was no evidence of congestive failure at the time of the pulmonary function studies. Electrocardiographic, X-ray and fluoroscopic studies were within normal limits. Patient SC had been in mild congestive heart failure but was not believed to be in failure at the time of the studies.

Fluoroscopic and X-ray examinations were carried out in all patients to determine whether there was tracheal compression by an enlarged thyroid gland. No definite tracheal compression was observed except in RR.

Methods. Inspiratory capacity, expiratory reserve volume, and vital capacity were measured with a 13 L spirometer. Distribution of inspired air was measured by the nitrogen meter single breath test (6). Maximal breathing capacity was measured by inspiring from a 120 L spirometer through a low resistance valve. Respiratory rate, tidal volume, and minute volume at rest and during the third to seventh minutes of exercise were measured using the 120 L spirometer. The minute volume was corrected to BTPS (body temperature and pressure saturated with water vapor). Exercise studies were performed by walking on a treadmill (slope 4¼°, speed 2.4 mph unless otherwise indicated). Ventilation during exercise was measured with the subjects inspiring air or oxygen. Arterial (brachial) blood samples were obtained while the subjects were breathing air at rest, air during exercise, and oxygen at rest. Arterial blood oxygen content, capacity, and carbon dioxide content were determined using the Van Slyke and Neill apparatus (7). Arterial blood pH readings were made at room temperature using a glass electrode and the correction factors of Rosenthal to convert to 37° C (8). Partial pressures of arterial blood carbon dioxide were calculated by applying the line charts of Van Slyke and Sendroy (9) to the pH and carbon dioxide content values obtained on whole blood.

Oxygen consumption and carbon dioxide production at rest and during the fourth and fifth minutes of exercise were measured by collection of expired gas in a Douglas bag and analysis in a Scholander 0.5 ml gas sample apparatus (10), with correction to 0° C and 760 mm Hg dry. Duplicate samples from each bag were analyzed and required to check within ± 0.05 per cent. Physiological dead space during resting and exercising ventilation was calculated by the Bohr formula (11).

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TABLE I

Physical characteristics, laboratory data before therapy, and results of therapy

Patient	Sex	Age	Ht	Wt	Basal metabolic rate	Radioactive iodine uptake	Protein-bound iodine	Dyspnea*	Antithyroid treatment	Clinical status post-therapy
		yrs	*in*	*lbs*	%	%	*μg/100 ml*			
RR†	F	41	62	131	+72		12.2	xxx		
DS	F	21	66	131	+28	77		xxxx	Propyl.‡ Surgery	Euthyroid
FC	F	41	65	173	+40	78	9.3	xxx	RaI§	Euthyroid
MC	F	52	68	131	+35	91	10.2	xx	RaI	Hypothyroid
LF	F	34	63	103	+52	87	11.0	x	Propyl. Surgery	Euthyroid
SC‖	F	45	64	148	+57	94	11.5	xxxx	RaI	Euthyroid
AT	F	42	68	159		67	11.6	xxxx		
EL	F	34	65	105	+ 3	58	6.5	xxx		
AL¶	F	55	62	107	+63	78	9.5	xxxx	RaI	Euthyroid
HR	F	46	65	124		88	7.5	x		
MB	M	43	73	157	+31	78	9.4	xxx		
EM	F	56	63	162	+70		14.0	x	Propyl.	Euthyroid
EQ	F	33	64	137	+28	59	6.7	xxxx		

* x = No change in exercise tolerance; xx = dyspnea on severe exertion; xxx = dyspnea on moderate exertion; xxxx = dyspnea on mild exertion.

† Episode of acute pulmonary edema 1 month prior to study and compression of trachea by enlarged thyroid observed on fluoroscopy.

‡ *n*-Propylthiouracil.

§ Radioactive iodine-131.

‖ Slight pulmonary congestion.

¶ Aortic stenosis, no clinical evidence of decompensation.

Functional residual capacity (FRC) and airway resistance were measured in the body plethysmograph (12, 13). Lung compliance was measured during normal ventilation by the method of Mead and Whittenberger (14). Maximal inspiratory and expiratory pressures at the mouth were measured against a closed airway using a capacitance manometer.

Apparent CO diffusing capacities (D_L)[1] were estimated by a modification of Krogh's single breath method described previously (15). Alveolar samples were analyzed for CO with an infrared analyzer,[2] and for He and O_2, with a continuous sampling mass spectrometer.[3] Plasma CO tension in equilibrium with mixed venous carboxyhemoglobin was estimated at the start and at the end of each series of breath-holding measurements with a rebreathing technique and subtracted from the alveolar CO tensions in a manner previously described (16) for estimating true CO tension gradients during breath-holding.

Diffusing capacity of the pulmonary membrane (D_M) and pulmonary capillary blood volume (Vc) were calculated from measurements of D_L at more than one alveolar oxygen tension using the following relationship derived by Roughton and Forster (17):

$$1/D_L = 1/D_M + 1/(Vc\theta). \quad [1]$$

D_L and D_M are expressed in ml/(min × mm Hg), and θ is the rate of CO uptake by the red cells in 1 ml of whole blood expressed as milliliters per minute per millimeter Hg plasma CO tension; Vc then is in milliliters. The reciprocal of θ was approximated from the alveolar oxygen tension ($P_{A_{O_2}}$) as follows (17):

$$1/\theta = 0.72 + 0.0058\ P_{A_{O_2}}. \quad [2]$$

The reciprocals of D_L for each subject were plotted against $1/\theta$ and the best straight lines to describe these relationships were determined by the method of least squares. Vc was calculated from the slopes and D_M from the intercepts of these lines at $(1/\theta) = 0$. Since the values of $1/\theta$ are based on an assumed oxygen capacity of 20 vol of gas per 100 ml of blood, estimates of Vc were corrected to the patient's oxygen capacity by multiplying the measured capillary volume by the ratio,

$$\frac{20}{\text{patient's } O_2 \text{ capacity (vol \%)}}.$$

Mean and instantaneous pulmonary capillary blood flows were measured in duplicate by the plethysmographic nitrous oxide method of Lee and DuBois (18).

All measurements at rest were made with subjects seated comfortably in a chair. Three to 16 months after therapy, 7 of the 13 patients were re-evaluated by the endocrine clinics of the University and Philadelphia General Hospitals and were restudied as described above.

[1] The subscript referring to carbon monoxide (CO) is omitted in this paper; D_L, D_M and θ refer to $D_{L_{CO}}$, $D_{M_{CO}}$, and θ_{CO}, respectively, unless specifically stated otherwise.

[2] Beckman Instruments, Inc., Fullerton, Calif.

[3] Consolidated Electrodynamics Corporation, Pasadena, Calif.

TABLE II

Lung volumes before and after therapy

Patient	Vital capacity		Residual volume		Functional residual capacity		Total lung capacity	
	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy
	ml		*ml*		*ml*		*ml*	
RR	2,580 (91)*		1,540 (95)		2,000 (88)		4,200 (99)	
DS [7]†	2,460 (67)	2,830 (77)	1,000 (67)	635 (43)	2,180 (94)	2,100 (90)	3,460 (71)	3,465 (71)
FC [6]	2,700 (83)	3,400 (105)	1,190 (66)	1,370 (77)	2,400 (86)	2,630 (94)	3,890 (81)	4,470 (93)
MC [4]	2,480 (77)	3,160 (97)	2,430 (135)	1,390 (77)	3,400 (121)	2,390 (85)	4,960 (104)	4,650 (97)
LF [5]	2,520 (81)	3,040 (97)	1,600 (104)	1,390 (90)	2,600 (116)	2,660 (118)	4,120 (93)	4,440 (101)
SC [3]	2,420 (81)	2,600 (87)	850 (51)	890 (53)	1,920 (81)	1,380 (58)	3,270 (74)	3,490 (79)
AT	2,230 (63)		1,235 (75)		1,870 (76)		3,465 (71)	
EL	3,090 (92)		1,070 (68)		2,480 (102)		4,160 (90)	
AL [3]	3,220 (122)	2,940 (111)	1,990 (120)	1,750 (106)	3,280 (100)	2,700 (88)	5,040 (123)	4,360 (107)
HR	2,590 (83)		1,590 (95)		2,590 (108)		4,180 (92)	
MB	4,625 (95)		1,945 (74)		2,685 (72)		6,570 (93)	
EM [3]	2,640 (100)	3,240 (123)	2,290 (129)	1,315 (74)	2,690 (80)	2,340 (69)	4,940 (118)	4,690 (112)
EQ	3,080 (95)		1,330 (85)		2,460 (106)		4,410 (97)	
Mean	2,820 (87)	3,030 (100)	1,540 (90)	1,250 (74)	2,500 (95)	2,315 (85)	4,380 (93)	4,220 (94)

* Per cent of predicted values in parentheses. Since the predicted values of Needham, Rogan and McDonald (20) were measured at ATPS and the measured values in our subjects were corrected to BTPS, 9% has been added to the predicted figures.
† Number of months between studies in brackets.

Calculations of standard deviations (SD) were performed by conventional methods. The *t* test was used to determine the probability that the observed changes were significant (19).

RESULTS

Lung volumes (*Table II, Figure 1*). The vital capacities of three of the hyperthyroid patients (DS, MC and AT) were significantly less than the predicted normal values of Needham, Rogan and McDonald (20). The other ten patients had vital capacities within the predicted normal range. The mean inspiratory capacity generally was decreased, and the mean expiratory reserve volume generally was greater than predicted, although the deviations from predicted values were not significant. Mean FRC, residual volume, and total lung capacity were within normal limits. It is noteworthy that 3 of 13 patients prior to therapy had total lung capacities less than 75 per cent of predicted values. On the other hand, AL had a total lung capacity of 123 per cent of the predicted value before therapy. The small variations observed are in reference to predicted values, but critical evaluation can be made only by measurement of the lung volumes following successful therapy (Figure 1). No correlation was found between the vital capacity or other lung volumes measured at rest and the dyspnea frequently observed on exercise. The mean vital capacity increased in seven patients who were restudied after treatment ($p < 0.02$). The inspiratory capacity was slightly increased ($p < 0.05$) and the residual volume was not significantly changed. The total lung capacity did not change. These results are similar to those of Richards, Whitfield, Arnott and Waterhouse (21).

Mechanics of breathing (*Table III*). Maximal breathing capacity was less than 80 per cent of predicted values in three patients, according to the prediction formula of Baldwin, Cournand and Richards (22). In contrast to the observation of normal "elastic resistance" in two hyperthyroid patients by McIlroy, Eldridge and Stone (23),

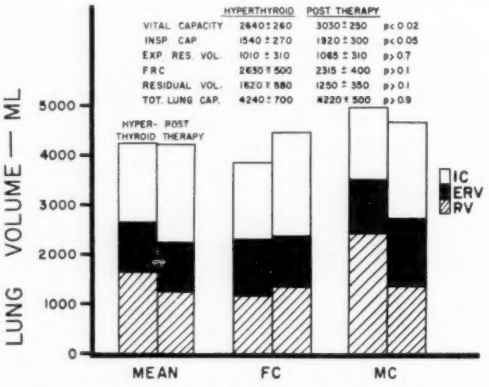

FIG. 1. MEAN LUNG VOLUMES AND STANDARD DEVIATIONS IN 7 PATIENTS BEFORE AND AFTER THERAPY. Patients FC and MC represent variations among group, FC showing an increase in inspiratory capacity post-therapy, and MC an increase in inspiratory capacity and a decrease in residual volume. IC = inspiratory capacity; ERV = expiratory reserve volume; RV = residual volume.

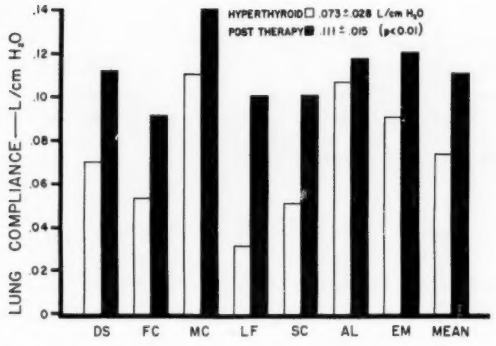

FIG. 2. LUNG COMPLIANCE PRE- AND POST-THERAPY IN 7 PATIENTS.

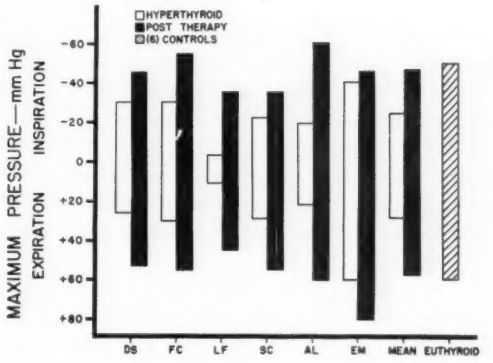

FIG. 3. MAXIMAL INSPIRATORY AND EXPIRATORY PRESSURES MEASURED AT THE MOUTH AGAINST A CLOSED AIRWAY PRE- AND POST-THERAPY.

lung compliance in the present study was lower than the predicted value in practically all the patients. Airway resistance was normal, except in one patient who had a large goiter and a small increase in airway resistance. Maximal pressures measured at the mouth on both inspiration and expiration against a closed airway were decreased significantly when compared to those measured on a control group of six female euthyroid subjects of similar age and body size. One patient, LF, had exceedingly low values of maximal inspiratory pressure before therapy.

After treatment, lung compliance increased to normal levels (Figure 2). Airway resistance measurements were unchanged when compared to the pre-therapy measurements. There were significant increases in maximal inspiratory and expiratory pressures measured at the mouth (Figure 3).

TABLE III

Mechanics of breathing

Patient	Maximal pressures: Inspiration, Before therapy	Maximal pressures: Inspiration, After therapy	Maximal pressures: Expiration, Before therapy	Maximal pressures: Expiration, After therapy	Maximal breathing capacity, Before therapy: *L./min*	Maximal breathing capacity, Before therapy: *% pred.*	Lung compliance, Before therapy	Lung compliance, After therapy	Airway resistance, Before therapy	Airway resistance, After therapy
	mm Hg						*L./cm H₂O*		*cm H₂O/L./sec*	
RR					59	70			2.94	
DS	30	45	29	53	72	68	0.070	0.112	1.98	1.70
FC	30	55	30	55	123	123	0.053	0.091	1.73	1.14
MC					59	75	0.110	0.140	1.69	1.45
LF	3	35	11	45	92	108	0.029	0.100	1.26	1.48
SC	22	35	29	55	86	100	0.052	0.100	0.90	1.30
AT	20		61		79	82	0.066		1.46	
EL	20		32		77	96	0.092		1.83	
AL	19	60	22	60	87	130	0.106	0.117	0.66	0.83
HR	40		55		78	98	0.090		1.32	
MB*	35		40		146	116	0.015		1.49	
EM	40	45	60	80	88	111	0.090	0.120	0.71	1.04
EQ	15		30		87	95				
Mean	24	46	35	58	86	98	0.076	0.111	1.50	1.27
SD	±11	±9	±16	±11			±0.025	±0.015	±0.60	±0.27
	$p < 0.01$		$p < 0.01$				$p < 0.01$		$p > 0.05$	
Normal	49		60				Female 0.09–0.19		0.7–2.4	
SD	±10		±17				Male 0.14–0.24			

* MB not included in the mean.

TABLE IV

Distribution of inspired air, ventilation, and oxygen consumption before therapy

Patient	Alveolar distribution	Minute volume		Frequency		O_2 consumption		O_2 extraction*	
		Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise
	% change (750–1,250 ml)	*L/min/m² BTPS*		*resp./min*		*ml/min/m² STPD*		*%*	
RR	1.0	6.2		21					
DS	1.7	4.4	30.3	16	33	160	515	3.92	4.55
FC	0.8	6.1	24.7	27	42		660		5.25
MC	0.8	6.7	17.8	25	30	208	710	4.10	4.00
LF	0.3	7.3	23.6	30	40	216	420	2.96	3.63
SC	1.5	6.4	27.1†	28	48	200	640	4.25	3.74
AT	0.2	6.4	36.2	29	54	212	810	4.20	3.26
EL	2.5								
AL‡	1.9	5.5		17		182		4.20	
HR	0.8	5.9	27.7	23	43	194	850	3.50	3.64
MB	0.5	8.8	30.1	32	49	175	496	2.50	4.65
EM	0.3								
EQ	0.3	5.6	17.4	25		179	530	3.80	3.62
Mean	1.0	6.3	26.1	25	42	191	625	3.71	4.04
SD		± .3	±5.7	±5	±8	±19	±139	±0.58	±0.6
Mean euthyroid controls§		4.5	12.5	19	20	138	523	4.07	5.51
SD		±0.2	±3.8	±6	±4	±22	±158	±0.94	±1.06
p Values		p<0.01	p<0.01	0.05>p>0.02	p<0.001	p<0.01	p>0.05	p>0.05	p<0.01

* Ventilation measured during period of oxygen consumption.
† 1 mph, 4½° slope.
‡ Could not tolerate exercise.
§ Average age 35 yrs; average weight 123 lbs.

Ventilation and gas exchange (*Table IV*). In all patients studied, distribution of inspired air was within normal limits. Frequency of breathing at rest was increased in hyperthyroid patients compared to that of a group of seven euthyroid females of similar body size and age. The hyperthyroid patients at rest had an increased minute ventilation per unit of body surface area (BSA) with decreased tidal volumes. The respiratory quotient of the hyperthyroid patients was within normal limits at rest. There was a significant increase in minute ventilation in the hyperthyroid patients during exercise on a treadmill (26.1 ± 5.7 L per minute per m²) compared to the minute volume of exercising euthyroid controls (12.50 ± 3.8 L per minute per m²). The increase in minute volume

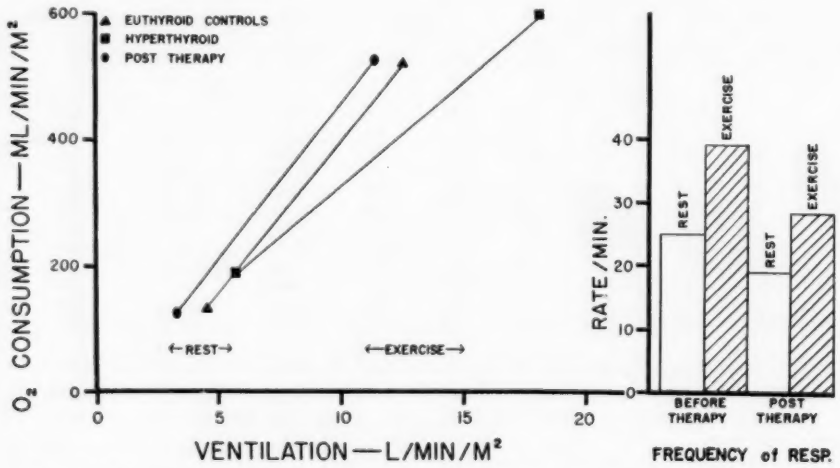

FIG. 4. OXYGEN CONSUMPTION, MINUTE VENTILATION, AND FREQUENCY OF RESPIRATION AT REST AND DURING EXERCISE IN EUTHYROID AND HYPERTHYROID SUBJECTS PRE- AND POST-THERAPY. The mean results for rest and exercise have been connected by straight lines.

TABLE V

Oxygen consumption and ventilation before (B) and after (A) therapy

Patient	Minute volume				Frequency				O_2 consumption				O_2 extraction*			
	Rest		Exercise		Rest		Exercise		Rest		Exercise		Rest		Exercise	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
	L/min BTPS				*resp./min*				*ml/min STPD*				%			
DS	7.50	5.33	50.0	21.3	16	13	33	23	265	254	850	932	3.92	4.25	4.55	5.50
FC	11.40	7.55	46.0	19.6	27	25	42			227	1,220	1,150		3.00	5.25	5.90
MC	8.30	3.50	28.8	24.2	25	15	30		340	138	1,160	1,065	4.10	3.94	4.00	6.10
LF	10.65	5.56	34.2	12.3	30	21	40	31	315	216	615	523	2.96	3.90	3.63	5.13
SC	8.00	4.47	49.6	22.0	29	22	48	31	342	210	1,100	1,030	4.25	4.70	3.74	5.65
AL†	8.03	6.90		43.5	17	16		24	265	254		770		3.16		
Mean	9.26	5.28	41.72	29.42	25	19	39	28	315	204	989	940	3.81	3.96	4.23	5.66
SD	±1.50	±1.34	±8.6	±4.1	±5	±4									±0.58	±0.36
	$p < 0.01$		$p < 0.01$												$p < 0.01$	

* Ventilation during period of oxygen consumption measurement.
† Unable to tolerate exercise while hyperthyroid.

during exercise was accomplished for the most part by an increase in frequency of breathing in the hyperthyroid subjects (42 ± 8; controls, 20 ± 4). At rest, mean oxygen consumption per unit BSA was significantly higher in hyperthyroid patients than it was in euthyroid controls, but during exercise oxygen consumption was not significantly different in the patient and control groups (Figure 4).

The oxygen extraction (rate of oxygen consumption divided by minute volume of ventilation) was not significantly different in the hyperthyroid patients and euthyroid controls at rest, but during exercise it was significantly less in the patients than in control subjects.

After treatment, frequency of breathing and minute ventilation during rest and exercise decreased significantly in the seven patients who were restudied (Table V, Figure 4). Oxygen extraction during rest was not significantly different after therapy, but during exercise this ratio increased significantly above the pre-therapy levels.

Dyspnea. The euthyroid subjects accomplished the required exercise without dyspnea. Preceding the exercise test, the hyperthyroid patients were instructed to signal by hand if they experienced respiratory discomfort. In four patients

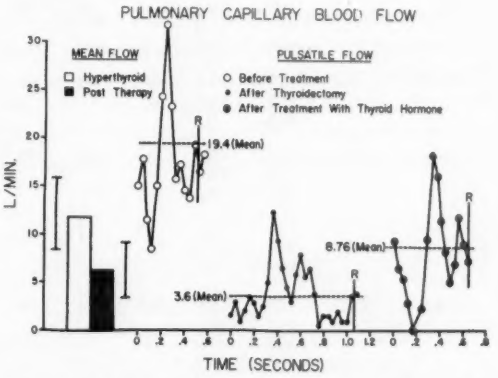

FIG. 5. MEAN PULMONARY CAPILLARY BLOOD FLOW BEFORE AND AFTER THERAPY (ON THE LEFT). Range bars define 1 standard deviation. Pulsatile pulmonary capillary blood flow of Patient DS appears on the right. Flow in liters per minute is on ordinate; time in seconds is on abscissa. 0–R (– – –) indicates one R–R interval on electrocardiogram; ○ = pulsatile flow in Patient DS before therapy; ● = after therapy and prior to clinical appearance of hypothyroidism; ⊕ = after treatment with thyroid hormone.

who so signalled, the inspiratory gas was changed from air to oxygen by manipulation of a hidden valve. In all four subjects dyspnea disappeared and there was a fall in rate of respiration. On re-examination, all patients had noticed improvement in exercise tolerance (Table V). AL, who was unable to tolerate the exercise test prior to therapy, stated that she could climb two or three flights of stairs without discomfort.

Pulmonary capillary blood flow and diffusing capacity. In the hyperthyroid patients at rest, peak levels of pulsatile flow in the pulmonary capillaries reached levels as high as those obtained on normal euthyroid subjects immediately after mild exercise (18). A representative pattern of pulsatile flow in Patient DS is shown in Figure 5. Following remission of symptoms, mean flow rates and peak flow rates decreased (Table VI and Figure 5). The low pulmonary capillary blood flow observed following therapy in three of the patients appears to be related to impending hypothyroidism, since these patients subsequently became grossly hypothyroid. In one of these latter three patients, DS, there was a substantial increase in pulmonary capillary blood flow during therapy with thyroid hormone (Figure 5). Apparent diffusing capacity, true membrane diffusing capacity and pulmonary capillary blood volume at rest were not elevated before treatment of hyperthyroidism and significant changes did not occur

TABLE VI

Pulmonary capillary blood flow ($\dot{Q}_c$), apparent diffusing capacity for CO(D_L) at different alveolar oxygen tensions, true membrane diffusing capacity (D_M) and pulmonary capillary blood volume (V_c) *

	Before treatment							After treatment						
				D_M		V_c					D_M		V_c	
Patient	$\dot{Q}_c$	$P_{A_{O_2}}$	D_L	Meas.	Pred.†	Meas.	Pred.†	$\dot{Q}_c$	$P_{A_{O_2}}$	D_L	Meas.	Pred.†	Meas.	Pred.†
RR		113	18.7	30	47	80	66							
		560	10.8											
DS	19.4	99	20.7	72	49	57	69	8.5	124	19.3	48	50	54	72
									230	15.2				
		362	13.0						403	11.9				
		622	8.0						574	9.1				
FC	7.5	105	18.3	34	56	59	80	10.4	120	16.9				
		216	13.2											
		504	9.5											
MC	9.6	112	20.4	48	48	65	68	4.3	144	19.6	63	51	62	73
		469	10.7						428	11.9				
		614	9.1						571	9.4				
LF	12.3	125	18.6	43	41	80	58	8.4	133	18.7				
		314	14.2											
		609	9.2											
SC	7.2	126	15.6	58	51	45	73	3.0	146	14.5	53	56	46	80
		308	10.2											
		609	6.5						581	6.7				
AL	14.0	126	14.7	65	41	50	58	3.1	120	12.4				
		248	11.3											
		423	9.0											
		568	5.8											
EM	12.2	120	14.3					6.2	120	15.0				
AT		129	22.5	56	55	75	79							
		317	16.4											
		608	10.8											
Mean	11.7			48	51	69	64	6.3			55	52	54	75

* Units: $\dot{Q}_c$ is in L/min; D_L and D_M are given in ml/(min × mm Hg); V_c is in ml; $P_{A_{O_2}}$ is given in mm Hg.

† Predicted D_M and V_c were calculated from predicted D_L utilizing the observation that the membrane and corpuscular resistances to CO diffusion in a normal resting subject are approximately equal (17). For this, D_L was predicted with a standard formula using body surface area (15).

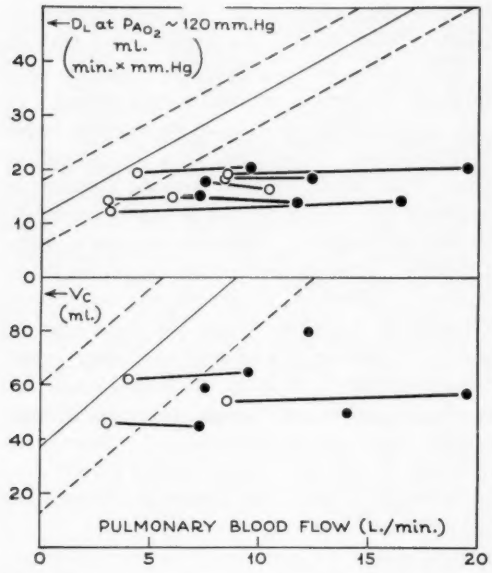

FIG. 6. THE RELATIONSHIPS OF LUNG DIFFUSING CAPACITY (D_L) AND PULMONARY CAPILLARY BLOOD VOLUME (V_c) TO PULMONARY BLOOD FLOW. Results for each patient before (●) and after (○) successful treatment of hyperthyroidism have been connected by straight lines. The solid diagonal lines are the regression lines describing the changes in D_L and V_c as blood flow was altered by physical activity in 4 normal male subjects (43); the parallel dashed lines delimit two standard errors of estimate on either side of the regression lines.

following successful therapy (Table VI). The absence of any significant correlation between changes of pulmonary blood flow and changes of either diffusing capacity or pulmonary capillary blood volume before and after treatment are illustrated in Figure 6.

Arterial blood gases (*Table VII*). The arterial oxygen saturation measured in seven of the hyperthyroid subjects was normal at rest and on exercise. There was not an abnormal right to left shunt as indicated by the normal amount of oxygen dissolved in plasma while breathing 100 per cent O_2. Arterial blood carbon dioxide tension and pH were within normal limits, but in several patients decreases in arterial blood P_{CO_2} occurred during exercise. During resting ventilation the total dead space comprised 38 per cent of the tidal volume, but during exercise, the total dead space increased to an average of 50 per cent in the four patients so studied.

DISCUSSION

Vital capacity

The present studies once again demonstrated a decreased vital capacity in some patients with hyperthyroidism. Although the mean pretreatment value was not significantly less than predicted, there were large rises of vital capacity in five of the seven patients restudied after therapy.

The low vital capacity prior to therapy may be related to the low lung compliance and to the muscular weakness which has been observed to accompany the hyperthyroid state (24). However, the fact that the total lung capacities of the group were the same before and after treatment is evidence against changes in lung compliance being the major determinants of the observed changes in vital capacity. An alternative explanation would be that sufficient effort to complete

TABLE VII
Arterial blood studies

Patient	Arterial oxygen saturation		Dissolved oxygen*	pH		$P_{A_{CO_2}}$	
	Rest	Exercise		Rest	Exercise	Rest	Exercise
	%		vol %			mm Hg	
DS	98.3	98.4	2.11	7.35	7.32	46	41
FC	95.1		1.77	7.36		42	
MC	97.5	98.7	2.00	7.40	7.33	42	43
LF	98.5	96.6	1.65	7.37	7.35	43	40
SC	94.8	96.1	1.93	7.37	7.38	46	39
AT	97.7	98.6	1.78	7.38	7.32	42	37
MB	99.0	98.3	1.90	7.34	7.41	46	36
Mean	97.3	97.8	1.76	7.37	7.36	44	39
Normal	96–99		>1.5	7.34–7.44		35–45	

* After breathing 99.6% oxygen for 15 minutes.

maximal expiration could not be sustained against *1*) the opposing elastance of the rib cage, *2*) frictional forces in the lung and chest wall, and *3*) increased inspiratory muscle tone (25). Although it is possible that congestion of the lungs with blood is a major factor causing the low vital capacity, the application of tourniquets on several of the hyperthyroid subjects did not elevate vital capacity to predicted or post-therapy levels. Since airway resistance measurements were normal in all but one of the patients, it is unlikely that generalized bronchial obstruction played a prominent part in the observed changes in vital capacity. RR, who had a large goiter with tracheal compression, had a slight increase in airway resistance, which did not decrease after an aerosolized bronchodilator and was therefore probably not due to bronchospasm. The normal values for distribution of inspired air in these patients also is not consistent with diffuse obstructive pulmonary disease.

Compliance

The mechanism of the decreased lung compliance observed in the hyperthyroid patients is not clear at the present time. Five possible mechanisms have been considered: *1*) changes in the FRC; *2*) pulmonary congestion and edema; *3*) increased intrathoracic blood volume; *4*) alterations in the retractile properties of alveolar septa; *5*) a decrease in the number of functioning alveolar units during quiet breathing.

1) *FRC.* A marked decrease or elevation of FRC may affect the lung compliance (26, 27). A decrease in FRC may be related to closure of a sufficient number of alveolar units so that an increased "opening pressure" is required (28). An increase in FRC, on the other hand, may be accompanied by an increase or decrease in the lung compliance. If the FRC be sufficiently increased to expand the volume to a position on the pressure-volume curve near its plateau, the compliance would decrease. If, after therapy, the FRC were increased sufficiently so that previously collapsed alveoli were expanded, the compliance would increase. Analysis of our data, however, reveals no significant change in mean pre- and post-treatment FRC to account for the observed changes in lung compliance.

2) *Pulmonary capillary congestion and edema.* We have demonstrated that the peak pulmonary capillary blood flow of the hyperthyroid patients at rest reaches levels greater than that in most euthyroid subjects after moderate exercise. At peak levels of flow, the hydrostatic pressure in the capillaries may force fluid into the interstitial spaces from which colloidal osmotic pressure or lymphatic flow cannot remove it rapidly enough to prevent accumulation. Such an increase in interstitial fluid, although this is pure conjecture, might produce a decrease in pulmonary compliance. Evidence to implicate ventricular failure in the genesis of pulmonary edema in exercising hyperthyroid subjects is lacking. Bishop, Donald and Wade (29) measured "wedge" pressures in three resting hyperthyroid subjects and observed an elevation in only one subject without overt congestive heart failure. Our finding of a normal D_M and a normal Vc during rest in these hyperthyroid patients makes the presence of pulmonary edema seem unlikely. Furthermore, in 11 of the 13 hyperthyroid subjects, there was no clinical or X-ray evidence of pulmonary congestion and edema. The normal arterial oxygen saturations at rest and on exercise and the normal airway resistance measurements also would be inconsistent with the presence of pulmonary edema and congestion as causative factors in the decreased compliance.

3) *Changes in intrathoracic blood volume other than in pulmonary capillaries.* An increase in total blood volume has been previously observed in hyperthyroidism (30). However, an increase in intrathoracic blood volume displacing pulmonary gas does not seem likely as a factor in the observed decrease of compliance in view of the normal values for total lung capacity and the failure of tourniquets to elevate the vital capacities to predicted or post-therapy levels in these patients.

4) *Alterations in the retractile properties of alveolar septa.* These alterations might occur as the result of changes in composition, structure, or configuration of the elastic components of the alveolar septa (31). However, evidence relating to such changes caused by hyperthyroidism is not available. Changes in composition of the alveolar mucoid film (32, 33) might alter tension at the air-liquid interface of alveoli and of small airways. This also could significantly alter the retractile forces in the lung. Depositions of mucoid mate-

rials have been observed in the orbit and lower extremities of hyperthyroid patients, although there have been no observations of a change in the alveolar mucoid film in hyperthyroidism.

5) *Alterations in the number of functioning alveolar units.* Lung compliance is the sum of the compliance of the smallest functioning volume units. Occlusion of unstable alveolar units by interfacial forces might result from muscle weakness in hyperthyroidism. Such changes would produce a decreased compliance. Similar decreases in lung compliance have been observed in patients with poliomyelitis (34).

Exercise ventilation and oxygen consumption

The hyperthyroid subjects had significantly greater respiratory minute volumes and rates of respiration during exercise in comparison with post-therapy studies or measurements on euthyroid controls. These findings could be the result of several factors. *1*) Muscular weakness and decreased lung compliance may induce a rapid rate of respiration as a means of minimizing the work necessary to achieve a given ventilation (35, 36). *2*) An abnormally large production of carbon dioxide during exercise may act as a respiratory stimulant. Although these studies do not preclude an increased sensitivity of the respiratory centers to carbon dioxide in hyperthyroid patients, carbon dioxide production and arterial P_{CO_2} during moderate exercise were no greater than they were after therapy or in euthyroid controls. *3*) Increased heat may have been a stimulus to the respiratory center. An increase in rate of respiration and ventilation usually accompanies an increase in body temperature (*37*). Maley and Lardy (38) and Hoch and Lipmann (39) have demonstrated by *in vitro* techniques that thyroxine produces a biochemical alteration whereby energy in the cell is dissipated as heat rather than by the formation of high energy phosphates. Heat intolerance is a frequent clinical observation in hyperthyroid patients, and many of the patients studied complained of warmth and sweating during the exercise test. *4*) The respiratory center may be stimulated directly by thyroid hormone or its metabolites. Williams, Winters, Clapp and Welt (40) have recently observed an increase in ventilation in animals treated with dinitrophenol. In their experiments, hyperventilation occurred even in the presence of respiratory alkalosis. They postulated that dinitrophenol might act as a respiratory stimulant. Since thyroxine and dinitrophenol have many similar actions, it is possible that thyroid hormone may also exert a direct or indirect effect on the respiratory center. *5*) The subjects who observed dyspnea on exercise experienced rapid relief of this symptom when allowed to breathe 100 per cent O_2 while continuing the exercise, even though arterial oxygen saturation was normal prior to oxygen. Ventilation per unit of oxygen consumption may be decreased when 100 per cent O_2 is administered to certain types of patients, usually those with hypoxia. This may indicate that tissue demand for oxygen may be operative in the increased ventilation observed in patients with thyrotoxicosis.

The decreased oxygen extraction in exercising hyperthyroid patients is probably due to an increase in total dead space ventilation produced by an increased frequency of breathing and an augmented alveolar or parallel dead space ventilation. Similar results in exercising hyperthyroid patients have been reported by Bates (41). Alveolar ventilation appears to have been adequate during exercise as there was no evidence of arterial oxygen unsaturation or carbon dioxide retention in any of the patients studied.

The dyspnea observed in many of the patients, while they were hyperthyroid, did not appear to be related to the size of the goiter or to an increase in minute ventilation accompanying an increase in oxygen uptake (23), but rather to a combination of several factors. In no patient was the airway resistance increased to a level considered sufficient to cause dyspnea of airway obstruction, and the increment of minute ventilation during exercise was consistently greater than that of oxygen consumption. Therefore, the dyspnea of hyperthyroidism appears to be related to other factors such as decreased compliance, decreased respiratory muscular strength (42), increased dead space ventilation, and perhaps an abnormal stimulus to respiration.

Diffusion and capillary blood flow

In each of these hyperthyroid patients the dimensions of the pulmonary capillary bed, as reflected by D_L, D_M and Vc, were essentially normal at rest even though the resting blood flow was

approximately twice to three times normal. Bates has made similar observations in patients with hyperthyroidism during light exercise, showing that DL is low in respect to the oxygen consumption (41), and by inference, even lower in respect to the cardiac output.

These findings in hyperthyroid patients are not inconsistent with reported findings in other circumstances where pulmonary blood flow is increased and DL is not. Ross, Frayser and Hickam (43) have shown that increases of pulmonary blood flow induced by epinephrine or norepinephrine are not accompanied by changes of diffusing capacity; and Turino, Brandfonbrener and Fishman (44) have shown that diversion of the total resting cardiac output through one lung in a man does not increase DL in the over-perfused lung. Therefore, failure to find DL increased during rest in these hyperthyroid patients in response to the increased resting pulmonary blood flow does not necessarily indicate an abnormal response of the vascular bed.

These observations in hyperthyroid patients are of interest because of the still pending question as to which factors control the size of the pulmonary capillary bed. It may be that the pulmonary intravascular pressures are not sufficiently increased by the increased blood flow in hyperthyroidism to cause the capillaries to expand. The data of Bishop and associates (29) indicate that the relationship between pulmonary blood flow and pulmonary arterial pressure is normal in patients with hyperthyroidism, but significant data are not available concerning pulmonary capillary pressure. Thus, the possibility that lung capillaries have failed to expand in these patients, owing to a transmural capillary pressure lower than normal in respect to blood flow, cannot be eliminated. Furthermore, if the low lung compliances observed in these hyperthyroid patients reflect a true change in the elastic properties of the septal tissues of the lung, it is not unreasonable to suspect that septal capillaries are similarly affected. This would mean that the vascular volume would tend to be less than normal for the same transmural pressure difference.

In contrast to the findings in hyperthyroid patients, a close reproducible relationship has been found between pulmonary capillary blood flow ($\dot{Q}c$) and DL in normal subjects at rest and during various grades of exercise (45, 46). It is reasonable to suspect that DL is increased during exercise by passive distention of the capillary bed owing to an increase in intravascular pressure accompanying the increased flow. The observation that an increase in intravascular pressure at constant pulmonary blood flow causes DL to increase in the isolated perfused cat lung (47) confirms this explanation. However, the increase of DL during exercise in normal subjects may be caused by a mechanism or mechanisms other than passive distention of capillaries: *1)* The size of the pulmonary capillary bed may actually remain fixed during exercise but an apparent change of DL, DM and Vc could occur if the relationship between intracorpuscular oxygen tension and the reaction rate (θ) between CO and oxyhemglobin were altered during physical activity. This possibility has not been entirely disproved, but it seems unlikely, since θ is relatively insensitive to the changes of blood pH, CO_2 tension and lactic acid concentrations normally associated with exercise (48–50). *2)* Increased ventilation has been suggested as the principal cause for the increase of DL during exercise (43, 44). Hyperventilation at rest causes DL to increase significantly when measured by steady state methods, but hyperventilation cannot explain the increases of DL, DM and Vc during exercise measured by breath-holding methods (15). The increase of DL by steady state measurements during hyperventilation may be related in part to an associated increase of mean lung volume as suggested by the work of Marshall (51). *3)* The size of the pulmonary capillary bed may be primarily determined by vasomotor activity in small pulmonary blood vessels. For instance, changes in chemical composition of venous blood returning to the lungs during exercise might mediate the observed expansion of the pulmonary capillary bed either by a direct action on the blood vessels or by reflex vasodilatation. In support of this, inhalation of CO_2 by normal subjects has been found to increase DL before significant rises in pulmonary capillary blood flow can be demonstrated (52). In patients with hyperthyroidism, arteriovenous oxygen differences are smaller than normal, therefore, CO_2 tension in mixed blood may be normal or low, providing no stimulus for capillary bed expansion. This possibility requires further evaluation.

TABLE VIII

Right ventricular stroke volume, pulmonary capillary blood volume, and mean transit time for blood through pulmonary capillaries

Patient	Before treatment			After treatment		
	Stroke vol.	V_c	Mean capillary transit time	Stroke vol.	V_c	Mean capillary transit time
	ml	*ml*	*sec*	*ml*	*ml*	*sec*
DS	164	57	0.18	106	54	0.38
FC	62	59	0.47	120	59*	0.34
MC	120	65	0.41	67	62	0.86
LF	111	80	0.39	73	80*	0.57
SC	63	45	0.38	46	46	0.92
AL	127	50	0.21	39	50*	1.01
EM						
Mean	108	59	0.34	75	58	0.68

* These values were assumed to be the same after treatment as they were before treatment.

Transit times through pulmonary capillaries

Another aspect of these data which stimulates speculation concerns the short intervals that red cells apparently remained in lung capillaries of these hyperthyroid patients during one circulation (Table VIII). In normal subjects at rest the average time spent in pulmonary capillaries during a single circulation is about 0.75 second and, during moderate exercise, expansion of the capillary bed tends to keep transit time at 0.5 second or more even though blood flow may be tripled (43). Also, the pulmonary capillary blood volume in normal subjects tends to be larger than the cardiac stroke volume and this insures a fairly uniform time of exposure of red cells to the lung diffusing surface, despite the pulsatile nature of pulmonary capillary flow. In the hyperthyroid patients, the rate of blood flow during rest was doubled or tripled, whereas the volume of the pulmonary capillary bed remained fixed at normal resting dimensions and the capillary blood volume was less than the cardiac stroke volume (Table VIII). Therefore, the mean pulmonary capillary transit time was not only short in these patients, but some red cells must have flowed past the alveolar capillary diffusing surface in much less than the estimated mean transit time, as indicated in the lower half of Figure 7. As a consequence, the oxygen tension gradient between alveolar air and end-capillary blood in the lungs of these patients should have been larger than in normal subjects under the same conditions. In order to estimate the possible magnitude of this increased gradient, approximations of the time course of intracorpuscular oxygen tension during capillary transit in the lung have been constructed for Subject DS, using the Bohr integration procedure (53) (Figure 8). In order to construct these curves, D_L was estimated with Equation 2, assuming that the D_M for oxygen is 1.23 times that for CO and assuming two different average values for θ_{O_2}. Only recently, θ_{O_2} has been measured within the physiological range of oxygen saturations, and although it has been found to remain relatively constant within the range of zero to 85 per cent blood oxygen saturation, θ_{O_2} falls as the oxygen saturation rises above 85 per cent and approaches zero as oxygen saturation approaches completion (54). Assuming that the average θ_{O_2} for blood traversing the

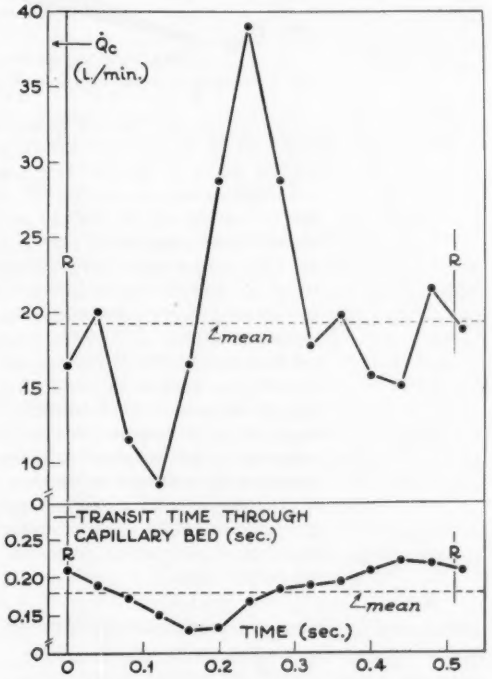

FIG. 7. ESTIMATES OF INSTANTANEOUS FLOW ($\dot{Q}c$) AND TRANSIT TIME THROUGH THE CAPILLARY BED OF RED CELLS ENTERING LUNG CAPILLARIES AT DIFFERENT MOMENTS WITHIN A CARDIAC CYCLE. The subject is DS before treatment of the hyperthyroid condition. The temporal positions of the ECG R waves in relation to flow events are indicated by vertical lines. In making estimates of transit time it was assumed that the pulmonary capillary blood volume remained 57 ml throughout the cardiac cycle (Table VI).

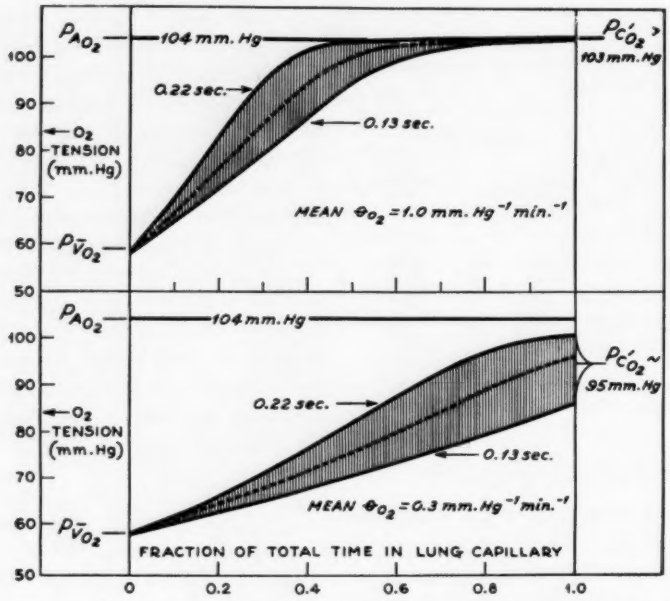

FIG. 8. OXYGEN TENSION IN RED CELLS AS THEY PASS ALONG AN ALVEOLAR CAPILLARY IN SUBJECT DS BEFORE TREATMENT OF HYPERTHYROIDISM. Two limiting values of θ_{O_2} have been assumed. $P_{A_{O_2}}$ and $P\bar{V}_{O_2}$ are the alveolar and mixed venous oxygen tensions, respectively. The dashed curves represent the oxygen tension in a red cell traversing the alveolar capillary bed in the calculated mean transit time (0.175 second, see Figure 7), and the solid lines forming the upper and lower boundaries of the cross-hatched areas represent the oxygen tension in red cells traversing capillaries in the longest and shortest times, respectively, during pulsatile flow (Figure 7). Pc'_{O_2} represents the end-capillary blood oxygen tension after mixing has smoothed out temporal as well as regional non-uniformity of oxygen saturation. Because of the shape of the blood oxygen dissociation curve, the end-capillary oxygen saturation of blood which has remained in the lung capillaries for longer than the mean transit time cannot take up sufficient additional O_2 to make up for the lesser end-capillary O_2 saturation of that blood which has traversed the capillaries in less than the mean transit time. For this reason oxygen tension of the mixed end-capillary blood (Pc'_{O_2}) is less than the oxygen tension of blood which has passed through the alveolar capillaries in the mean transit time, unless, of course, the transit time is constant throughout the cardiac cycle.

capillary bed in DS might reasonably lie between 1.0 and 0.3 mm Hg^{-1} min^{-1} (17), the mean $D_{L_{O_2}}$ would lie between 28 and 11 ml (per minute $\times$ mm Hg), respectively. The curves in Figure 8 were constructed, using these assumptions. The transit times were obtained from the data plotted in Figure 7. The rates of O_2 diffusion at different points along a pulmonary capillary and, consequently, the shape of the O_2 tension rise with time are critically dependent upon the manner in which θ_{O_2} decreases as hemoglobin saturation increases, but even at the lower assumed value for mean θ_{O_2}, the mixed end-capillary O_2 tension in this patient should have been within 10 mm Hg of the alveolar oxygen tension. This end-capillary gradient is still compatible with the normal resting arterial oxygen saturations measured in these patients (Table VII).

SUMMARY

1. The vital capacity was decreased in some, but not all, of the hyperthyroid patients studied.

Following therapy, the vital capacity reached predicted normal or higher levels in the majority of patients restudied.

2. The lung compliance in hyperthyroidism was decreased.

3. Elevation of airway resistance observed in only one patient was insufficent to produce symptoms of respiratory obstruction.

4. Measurements of pressures at the mouth on maximal expiratory and inspiratory effort against a closed airway indicated weakness of the respiratory muscles.

5. On exercise the patients' ventilation was increased in excess of the oxygen uptake when compared to a group of controls of similar age and body size. This was apparently related to an increase in the dead space ventilation due to an increase in frequency of breathing and alveolar dead space ventilation.

6. Following remission of symptoms produced by surgical thyroidectomy, radioactive iodine, or propylthiouracil drugs, the previously described abnormalities reverted to normal levels.

7. In hyperthyroid patients, apparent diffusing capacity of the lung (D_L), pulmonary capillary blood volume (V_c), and membrane diffusing capacity (D_M) at rest were not elevated in the presence of significant elevations of pulmonary capillary blood flow. Reductions of pulmonary capillary blood flow following successful therapy were not associated with significant changes in D_L, V_c, or D_M.

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STUDIES OF GLYCOGEN METABOLISM IN LIVER GLYCOGEN DISEASE (VON GIERKE'S DISEASE): SIX CASES WITH SIMILAR METABOLIC ABNORMALITIES AND RESPONSES TO GLUCAGON *

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Glycogen disease of the liver (von Gierke's disease) is a rare and incompletely understood disorder of childhood. It is characterized by defective breakdown of liver glycogen to glucose. In most cases, excessive liver glycogen deposition occurs, reaching levels as high as 14 to 17 per cent of the wet liver weight (1, 2). Major clinical manifestations include hepatomegaly; fasting hypoglycemia, acidosis and ketosis; delayed growth and development, and increased morbidity with even minor infections. The most severely affected children have usually died during infancy. Histologic studies of autopsy or biopsy specimens of liver reveal essentially normal architecture, but hepatic cells contain excessive amounts of glycogen and often, fat. The glycogen is usually normal in structure (3) and can be broken down to glucose by homogenates of normal animal or human liver (2, 4, 5). Spontaneous breakdown of glycogen during *in vitro* incubation of liver tissue from affected children is deficient (2, 4, 6).

The currently accepted pathways of liver glycogen metabolism are summarized in Figure 1. It was formerly believed that glycogen was synthesized through the action of phosphorylase, the same enzyme that catalyzes its breakdown. However, it is now agreed that this reaction, which occurs readily *in vitro*, is not an important one *in vivo* (7), and that glycogen synthesis probably proceeds through the uridine-diphosphoglucose pathway described by Leloir and Cardini (8). Glycogen breakdown is mediated by phosphorylase and amylo-1,6-glucosidase ("debrancher enzyme"), with glucose-1-phosphate as the principal product. This is converted to glucose-6-phosphate in a reversible reaction catalyzed by phosphoglucomutase. Glucose is liberated from this intermediate by glucose-6-phosphatase. Small amounts of glucose are also formed directly from glycogen through the action of amylo-1,6-glucosidase at branch points in the glycogen molecule. Other metabolic pathways open to glucose-6-phosphate are partial oxidative degradation through the "hexose monophosphate shunt" and breakdown to pyruvate and lactate via the glycolytic pathway.

Intensive biochemical studies by several investigators have led to the subclassification of hepatic glycogen disease into several types, based on determination of the activity of certain hepatic enzymes. The clinical syndrome described above has been seen in association with deficiencies of glucose-6-phosphatase, amylo-1,6-glucosidase, or phosphorylase (9, 10). More recently, Hers reported a case which did not have a deficiency of any of these enzymes (11). Thus the problems of the fundamental pathophysiology and the subclassification of this syndrome are not yet completely resolved. However, all cases share a basic functional abnormality—defective breakdown of liver glycogen to glucose.

The clinical test most commonly employed in the diagnosis of hepatic glycogen disease consists of observation of the blood glucose response to injection of epinephrine or glucagon. Normal children or adults respond to these agents with a prompt elevation of the blood glucose level. Patients with liver glycogen disease, on the other hand, show no response or only small and delayed increases in blood glucose concentration (1, 2, 5,

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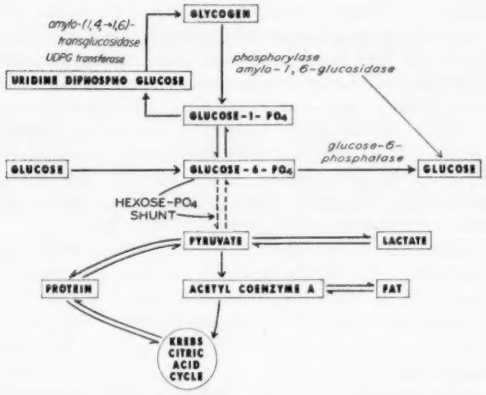

FIG. 1. CURRENTLY ACCEPTED PATHWAYS OF LIVER GLYCOGEN SYNTHESIS AND BREAKDOWN.

12–14). This has been considered as evidence that these hormones are ineffective or, at best, only partially effective in inducing hepatic glycogenolysis in this disease.

In the course of studies of glycogen deposition diseases, we have encountered six cases of liver glycogen disease with similar metabolic abnormalities and responses to glucagon. Observations in one of these cases (J.B.) were reported previously (15). The studies described below suggest that hepatic glycogenolysis occurred spontaneously in these patients in response to hypoglycemia and that injections of glucagon induced rapid breakdown of liver glycogen. However, the principal end-product of hepatic glycogenolysis appeared to be lactate rather than glucose.

METHODS AND CLINICAL MATERIAL

Glucose was determined by the Nelson method (16). Lactate was determined by the Barker-Summerson method (17) and by an enzymatic method using lactic dehydrogenase (18). Both procedures gave similar results, but since the latter is more specific and proved to be convenient and dependable, it was used for most of the determinations. Glycogen was determined by the method of Good, Kramer and Somogyi (19), using the Nelson technique for the determination of glucose after hydrolysis.

None of the cases under study showed evidence of heart disease, skeletal myopathy or cirrhosis of the liver. Their principal diagnostic features are presented in Table I. Additional details will be reported elsewhere (20). Brief clinical summaries follow.

Patient 1. J.B. was a 10 month old white male infant admitted for investigation of an enlarged liver and episodes of vomiting and irritability. Two older siblings had died in infancy with enlarged livers, and the diagnosis of hepatic glycogen disease had been established in one of them. Hepatic enlargement had been noticed during the first few months of the patient's life and had increased progressively until admission. The infant developed hypoglycemia, acidosis and ketosis rapidly on fasting. Blood glucose values as low as 10 to 16 mg per 100 ml were repeatedly obtained after 4 to 5 hours of fasting. Injection of epinephrine or glucagon

TABLE I

Principal diagnostic features of patients under study

Patient	Liver glycogen	Muscle glycogen	Liver* phosphorylase	Liver* glucose-6-phosphatase	Fasting hypoglycemia	Remarks
	%	%	*μmoles P/g/30 min*			
1	8.6	0.19	418	267	Severe	Liver and muscle incubation results presented in Table II
2	10.2	1.48	56	9	Moderate to severe	Incubation of isolated glycogen with purified phosphorylase resulted in 30% degradation
3					Moderate to severe	Liver biopsy not performed
4	9.5†				Mild	Liver glycogen 8.9% after 24 hours at room temperature†
5	7.8‡			Less than 10% of normal‡	Severe	
6	7.1	0.38	489	0	Severe	See text for liver incubation results

* Method of Cahill, G. F., Zottu, S. and Earle, A. S. Endocrinology 1957, **60**, 265. Normal values in our laboratory for human (adult) liver obtained at surgical biopsy are: phosphorylase, 200 to 500; glucose-6-phosphatase, 150 to 300.

† Personal communication, Dr. Harry W. Bain, Toronto.

‡ Reference 13.

produced slight or no rise in the blood glucose level. He had hyperlipemia. Laparotomy, and liver and muscle biopsy were performed. Histologic diagnosis was liver glycogen disease. Liver glycogen concentration was 8.6 per cent.

Patient 2. S. J. was a 2 year old white female infant admitted for investigation of a greatly enlarged liver. An enlarged abdomen was noted soon after birth. There was no familial history of glycogen disease. Moderate to severe hypoglycemia was noted after overnight fasting; on two occasions values below 20 mg per 100 ml were recorded. Mild acidosis and ketosis developed during fasting. Subcutaneous injection of epinephrine resulted in little or no rise of blood glucose (maximal increase, 16 mg per 100 ml). Injection of glucagon resulted in small and delayed rises in blood glucose. She had moderate hyperlipemia. Laparotomy, and liver and muscle biopsy were performed. Histologic diagnosis was liver glycogen disease. Liver glycogen concentration was 10.2 per cent.

Patient 3. Jay F. was a 6 year old white female admitted with the diagnosis of hepatic glycogen disease. An older sibling (Patient 4) also had the disease. Enlargement of the abdomen had been noticed at the age of 4 months, and increasing hepatomegaly was recorded from the age of 7 months. The patient's growth was retarded but her mental development was normal. She had been maintained in relatively good condition by very frequent feedings. However, her course was marked by episodes of infection, acidosis, and severe epistaxis. She was an abnormally small child for her age, with a greatly enlarged liver. She developed moderate to severe hypoglycemia on fasting, with blood glucose levels falling as low as 17 mg per 100 ml. Injection of epinephrine produced an insignificant rise in the blood glucose concentration. Injection of glucagon resulted in a small and delayed rise in the blood glucose level. Liver and muscle biopsies were not performed.

Patient 4. Jill F. was a 12 year old white female, the older sister of Patient 3. The diagnosis of hepatic glycogen disease had been established at the age of 17 months by clinical studies and liver biopsy. Injection of epinephrine resulted in a maximal rise of 12 mg per 100 ml in the blood glucose concentration. Histologic diagnosis was liver glycogen disease. Liver glycogen concentration was 9.5 per cent; there was only slight glycogenolysis in the biopsy specimen during 24 hours at room temperature. Her course since then has been characterized by frequent infections, bouts of acidosis, severe epistaxes and greatly retarded growth. Mental development was normal. She had a huge abdomen with massive hepatomegaly.

Patient 5. J.K. was a 6 year old white male. The diagnosis of liver glycogen disease had been established in the immediate neonatal period, when he was found to have hepatomegaly, severe hypoglycemia and acidosis. Intensive studies at that time (13), including liver biopsy, revealed the following. Moderate fasting induced severe hypoglycemia and acidosis, with occasional convulsions. There was little or no rise in blood glucose after injection of epinephrine or glucagon. Liver glycogen concentration was 7.8 per cent and hepatic glucose-6-phosphatase was greatly reduced. Histologic diagnosis was liver glycogen disease. One sibling had died in infancy of liver glycogen disease. The child's course since infancy was characterized by occasional episodes of severe hypoglycemia (despite a regimen of frequent feedings), hyperlipemia and xanthomata, and delayed growth. Mental development was normal. He was an abnormally small child (bone age 3.25 years) with a protuberant abdomen and a greatly enlarged liver.

Patient 6. M.M. was a 1 year old white female infant admitted with the diagnosis of liver glycogen disease. At 2 days of age she had been found to have hepatomegaly and a blood glucose level of 2 mg per 100 ml. Liver biopsy at 5 days of age revealed a histologic appearance typical of liver glycogen disease (no chemical determination of glycogen content). She was a small, weak infant, with a greatly enlarged liver, who developed severe hypoglycemia after 4 hours of fasting. Bone age was 6 months. There was virtually no rise in blood glucose concentration after glucagon injection. She had hyperlipemia. Repeat liver biopsy revealed a glycogen concentration of 7.1 per cent.

RESULTS

In vitro incubation studies. After the completion of preliminary clinical studies, laparotomy was performed in Patient 1. This patient, who tolerated fasting very poorly, received a slow infusion of glucose during the night before surgery. At laparotomy a piece of liver and a small sample of rectus muscle were obtained for *in vitro* study. The specimens were chilled rapidly and transported to the laboratory under refrigeration. Tissue slices were cut as rapidly as possible and added to flasks containing Krebs-Ringer phosphate buffer, pH 7.4, chilled to 2° C. The order of slices entering the duplicate incubation flasks was such that no systematic effect attributable to the time of slicing would occur; i.e., the pattern used was ab . . . ba rather than aabb . . . . A liver sample was withdrawn midway through the slicing procedure for determination of the initial glycogen concentration. Refrigeration of the incubation flasks was continued until slicing was completed. All flasks were then placed in a Dubnoff shaker-incubator and incubated for 2 hours at 37° C under a continuous flow of oxygen.

Liver slices were incubated in buffer alone and with several additives. At the end of 2 hours, an aliquot was withdrawn from each flask for glucose determination, 30 per cent potassium hydroxide was added to the incubation flasks, and glycogen

TABLE II

Results of in vitro liver slice incubations, (Patient 1)

Wt of slices	Type of incubation*	Glycogen	Glycogen decrease	Glucose recovered
mg		*mg glucose/g tissue*		
153	Not incubated; initial glycogen	86		
176	After incubation;	79	7	0
137	without additives	79	7	0
165	After incubation with	64	22	4
90	50 μg glucagon/flask	48	38	10
162	After incubation with	58	28	27
190	rat liver homogenate†	60	26	29

* All incubations conducted in 3 ml Krebs-Ringer phosphate buffer containing additives as described.

† Each flask contained 638 mg of rat liver homogenized in buffer. The results shown are after correction for incubated rat liver blanks, which were: glycogen 0, 0; glucose 4.9, 4.7 mg per g.

determination was performed. Muscle slices were incubated in Krebs-Ringer phosphate buffer without additives.

Table II presents the results of liver slice incubations without additives, with glucagon, and with a homogenate of liver obtained from a fasted normal rat. The initial liver glycogen concentration was 86 mg per g. Slices incubated without additives showed only a slight decline in glycogen content during incubation and no glucose production. Unequivocal stimulation of glycogenolysis was induced by glucagon. However, only small amounts of glucose appeared in the incubation medium. Incubation with rat liver homogenate, on the other hand, resulted in the production of glucose in amounts corresponding to the loss of glycogen.

In contrast to the defective glycogenolysis observed during incubation of liver slices, normal glycogen breakdown was observed during incubation of muscle. The initial muscle glycogen concentration was 1.9 mg per g, a relatively low value. After incubation it was 0.3 mg per g.

The failure to account for the products of glycogenolysis during incubation of liver slices was not anticipated and did not become known in time to permit analyses for other possible metabolites of glycogen. However, material from another incubation experiment, performed at the same time, was available. Liver slices had been incubated with C^{14}-glucose, without hormonal additives. The flask contents had been frozen and stored for later study. This material was thawed and analyzed for glycogen, glucose and lactate. The amount of glucose recovered was 0.59 mg per flask, virtually the same as the amount added—0.54 mg. Glycogen concentration was 68 mg per g, indicating breakdown of 18 mg per g, more than was observed in the control incubations shown in Table II. The amount of lactate recovered was equivalent to 21 mg per g of liver. Determination of radioactivity demonstrated that very little of this lactate could have been derived from the added glucose. Although the original concentration of lactate in this liver specimen had not been determined, it is most unlikely that it could have been high enough to account for the amount recovered (about 2,600 mg per 100 ml of liver water). Thus, it must be concluded that the lactate recovered was formed during incubation from precursors in the liver slices, and it appears likely that it arose from liver glycogen. No data are available on lactate production during incubation with glucagon.

In vitro incubation of liver slices was also performed in Patient 6. In this case, however, no effect of glucagon could be demonstrated. The initial glycogen concentration was 71 mg per g (individual specimens, 72 and 69). After 1 hour of incubation in Krebs-Ringer phosphate buffer, the glycogen concentration was 65 mg per g (specimens 68, 60, 70, 58, 68). After incubation with glucagon, it was 63 mg per g (specimens 61, 64, 55, 72). Both in the control and the glucagon incubations, lactate was the principal metabolite recovered. Initial concentrations of glucose and lactate in the liver slices were 0.7 and 0.9 mg per g, respectively. An average of 1.5 mg per g of glucose and 2.3 mg per g of lactate appeared during incubation.

Blood lactate studies. Marked elevations of fasting blood lactate levels were observed in all six children. These are shown in Table III. The lowest value recorded was 30 mg per 100 ml, observed once in Patient 2. On numerous other occasions, however, this child had higher levels, and her average fasting blood lactate concentration was 86 mg per 100 ml. In contrast, among 52 consecutive determinations in healthy controls and patients with various other diseases, including both infants and adults, the highest value observed was 26 mg per 100 ml (average, 13).

Numerous determinations of blood lactate concentration were performed in five of the patients

TABLE III

Levels of lactate in blood of fasting subjects

Patient	No. of determinations	Lactate	
		Range	Mean
		mg/100 ml	
1	18	40–128	93
2	27	30–130	86
3	8	55– 85	66
4	1	69	
5	10	72–122	88
6	4	52–130	105
43 healthy and sick adults and children without glycogen disease	52	3– 26	13

at various time intervals after feedings, glucose infusions and other test situations. Although there were occasional anomalous findings, a consistent pattern emerged from these studies. The blood lactate level was high after periods of fasting and sustained hypoglycemia. It usually began to decline about an hour after hypoglycemia had been corrected. Glucose infusions usually produced greater and more rapid declines in lactate than did feedings. This fall in circulating lactate concentration often continued while the blood glucose level declined to hypoglycemic values, and the lowest blood lactate value was frequently obtained during a period of distinct hypoglycemia. However, after hypoglycemia had persisted for about an hour, a sharp rise in blood lactate concentration uniformly occurred. Figures 2 and 3 show blood glucose and lactate curves in association with glucose infusion or feeding. Figure 2 demonstrates reduction of lactate to normal levels after 3 hours of glucose infusion. The drop in lactate shown in Figure 3 was more rapid than that observed in most of these children after feedings; lactate rebound after 1 to 2 hours of hypoglycemia is well demonstrated.

The possibility was considered that endogenous epinephrine release secondary to hypoglycemia, and resultant glycogenolysis in muscle, might account for a major portion of the elevation in fasting lactate. In order to assess the contribution of this mechanism, epinephrine was administered to two patients at a time when their blood lactate levels were relatively low. It was reasoned that if endogenous epinephrine release played a dominant role in the production of the fasting hyper-

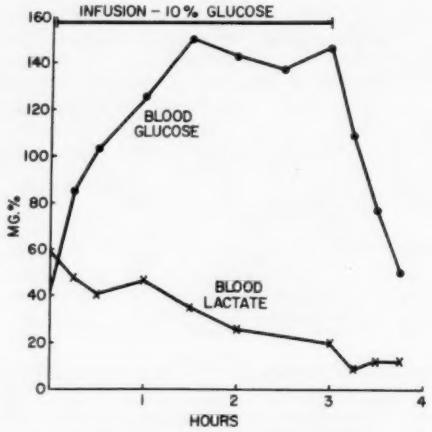

FIG. 2. RESPONSE OF THE BLOOD LACTATE LEVEL TO GLUCOSE INFUSION. Patient 2 (S.J.). Glucose infusion, 0.5 g per kg per hour, was administered after an overnight fast.

lactacidemia, administration of exogenous epinephrine should be able to reproduce this finding. Accordingly, after a suitable overnight fast, these children were given an infusion of 10 per cent glucose. After 3 hours of glucose infusion, which resulted in a substantial drop in lactate level, each child received a subcutaneous injection of epinephrine, 0.03 mg per kg. In Patient 1, the glucose infusion was continued; in Patient 2, the infusion was terminated at the time of epinephrine injection. The blood lactate values before and after

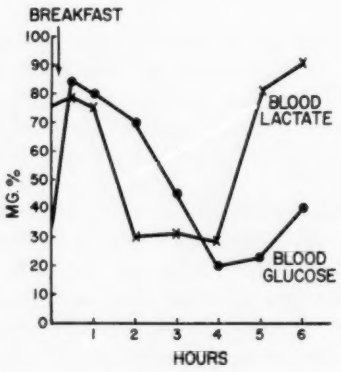

FIG. 3. RESPONSE OF THE BLOOD LACTATE LEVEL TO A FEEDING. Patient 3 (Jay F.). Overnight fast. Initial values, just before breakfast (fruit juice, eggs, milk, and bread; protein 20 g, fat 20 g, carbohydrate 60 g; calories 500).

TABLE IV

Effect of epinephrine on blood lactate levels of two patients

Schedule		Lactate	
		Patient 1	Patient 2
		mg/100 ml	
0 hr	Fasting	94	108
0–3 hr	Glucose infusion, 0.5 g/kg/hr		
3 hr	Just before ephinephrine injection, 0.03 mg/kg	47	38
	15 min after epinephrine		52
	30 min after epinephrine	38	47
	45 min after epinephrine		43
	60 min after epinephrine	47	43

epinephrine injection are shown in Table IV. There was no rise in Patient 1 and a modest rise in Patient 2. These results were consistent with the muscle biopsy findings previously obtained (Table I); Patient 1 had an unusually low level of muscle glycogen, while Patient 2 had a normal muscle glycogen concentration. Although the possibility that these children may be unusually resistant to the metabolic effects of exogenous epinephrine cannot be excluded, this experiment indicates that epinephrine release plays, at best, a relatively minor role in the production of the very high blood lactate levels described above. In contrast, when these children were given injections of glucagon under similar circumstances (see below), striking increases in lactate were always observed.

In vivo effects of glucagon. After the demonstration of an *in vitro* glucagon effect in Patient 1, clinical studies with this agent were performed in all six children. Injections of glucagon produced major rises of blood lactate concentration in all experiments. Figure 4 shows the responses of these patients to intramuscular injection of 1.0 to 2.0 mg of glucagon (0.1 mg per kg) during the fasting state. For comparison, the responses of four normal children of similar weights are shown. A striking difference between the two groups was observed. The normal children had sharp rises in blood glucose levels, but little change in circulating lactate. The patients with glycogen disease, on the other hand, showed sharp increases in lactate and abnormally small rises in glucose concentration. The blood lactate curves among these patients resembled the blood glucose curves of the controls, though the absolute rises were smaller.

Table V lists the average changes in blood glucose and lactate values during the first 30 and 60 minutes of glucagon action among the six patients and the controls. Although there was variation among the patients, the pattern for each patient was a consistent one. For example, Patient 1 showed no rise or only a slight rise in the blood glucose level after glucagon injection. On the other hand, Patients 2 and 3 always showed some increase in the blood glucose level, although it was not nearly so rapid nor so great as that seen among the controls.

The effect of glucagon was also examined at various intervals after feeding or glucose infusion in five of the patients. Sharp rises in the blood lactate level were obtained under all circumstances. The effect of glucagon was more dramatic if it was injected at a time when the blood lactate level was relatively "low." For example, Patient 6 had a rise in lactate of only 20 mg per 100 ml when the initial level was 122 mg per 100 ml (Figure 4, Table V). On another occasion, glucagon was given when the blood lactate level was 57 mg per 100 ml, after feeding. The lactate rose to 113 mg per 100 ml in 30 minutes. On a third occasion, when the initial lactate concentration was 19 mg per 100 ml, an increase to 119 mg per 100 ml was observed 1 hour after glucagon injection. Similar results were obtained in the other children.

Since doses of epinephrine presumably sufficient to induce glycogenolysis in muscle failed to reproduce the observed elevations in lactate (Table IV), and since glucagon exerts little or no effect on muscle, it appeared likely that the liver was

TABLE V

Mean changes in fasting blood glucose and lactate levels after glucagon injection (patients and controls)

Patient	No. of studies	Increase in glucose		Increase in lactate	
		0–30 min	0–60 min	0–30 min	0–60 min
		mg/100 ml		*mg/100 ml*	
1	2	1	9	42	87
2	3	15	30	36	52
3	3	18	36	54	55
4	1	13	35	64	45
5	1	6	6	35	31
6	1	4	5	20	13
Average, Patients 1–6		10	20	42	47
Average, 4 normal children		86	91	3	2

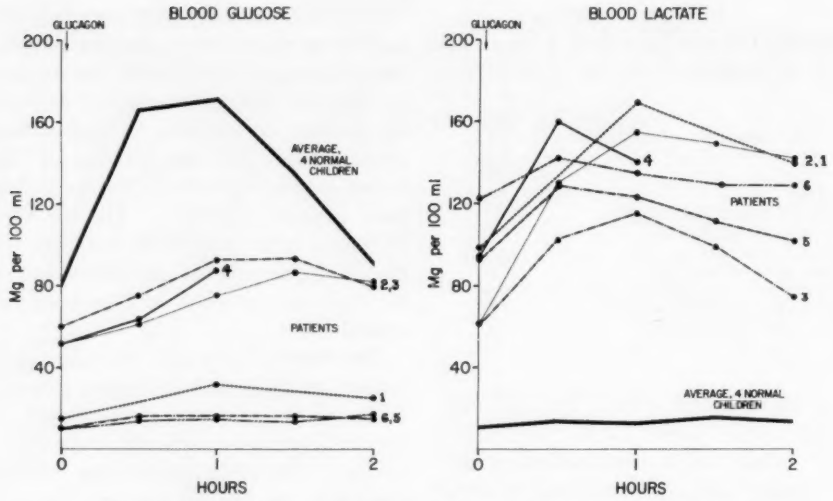

FIG. 4. EFFECT OF GLUCAGON INJECTION ON THE BLOOD GLUCOSE AND LACTATE LEVELS OF PATIENTS AND CONTROLS. The numbers to the right of individual curves identify the patients.

the source of the excess circulating lactate. We attempted to confirm this by an experiment based on the report of Mason and Andersen (21), who found that arterial lactate was higher than venous lactate in a child with glycogen disease. In normal individuals, in whom muscle is a principal source of circulating lactate, the concentration of this metabolite is higher in venous than in arterial blood. Venous (medial cubital vein) and capillary blood samples were obtained in Patient 3 before and after glucagon administration. The lactate concentration rose sharply after glucagon injection, as in Figure 4. However, we did not observe a significant capillary-venous lactate difference either prior to or 30 minutes after glucagon injection. This indicates that muscle was not contributing lactate to the circulation, but does not constitute as striking evidence for the hepatic origin of the lactate as does the observation of Mason and Andersen.

The acute effect on liver size of relatively large doses of glucagon was studied in Patients 1, 2, 3, 5, and 6. These children received 1 to 2 mg of glucagon per hour for 4 hours, by intravenous infusion. A decrease in liver size was observed in all. This became apparent after the first hour and was maximal after 2 to 3 hours, at which time liver margins were 2 to 4 cm above the initial levels in Patients 1, 2 and 3, and 1 to 2 cm above in Patients 5 and 6. Moderate to severe side effects were seen at this dose range, however. All five children developed restlessness and tachypnea and one vomited during the infusion. The clinical impression of acidosis was confirmed by determination of pH and bicarbonate content of blood in one patient. Distinct but relatively small changes in liver size were sometimes also observed after single subcutaneous injections of 1.0 to 1.5 mg of glucagon. These doses produced few or no side effects.

DISCUSSION

The patients discussed herein were heterogeneous with respect to hepatic enzyme assay. They include two "classic" cases of glucose-6-phosphate deficiency, one case without a demonstrated enzymatic defect, perhaps similar to that reported by Hers (11), and one case with a previously undescribed enzymatic pattern—a deficiency in both phosphorylase and glucose-6-phosphatase. A detailed discussion of the significance of these enzyme patterns and of the subclassification of "von Gierke's disease" is beyond the scope of this paper. It must be emphasized, however, that these children form a homogeneous group in other respects. They had greatly enlarged livers containing excess glycogen; fasting hypoglycemia, acidosis, and ketosis; hyperlipemia, and delayed growth.

Neither glucagon nor epinephrine evoked a hyperglycemic response. Spontaneous *in vitro* breakdown of glycogen to glucose was defective in the patients in whom this was studied. They had similar metabolic abnormalities and similar responses to glucagon. Thus, it seems appropriate to consider them together despite the differences in hepatic enzyme patterns.

Unequivocal stimulation of glycogenolysis by glucagon was demonstrated during *in vitro* incubation of liver slices in Patient 1. The principal end-product of this glycogenolysis was not glucose; indirect evidence suggests that it was lactate. In all six patients of this group, glucagon administration was followed by marked rises in blood lactate concentration. These increases in lactate resembled the increases in blood glucose concentration following the administration of glucagon to normal children. Comparison of capillary and venous lactate levels in one case indicated that the increment in lactate did not come from muscle. In all five children given glucagon infusions, an acute decrease in liver size was observed. Taken together, these findings suggest strongly that glucagon exerted its usual glycogenolytic effect on the liver in these cases, but that the principal end-product of glycogen breakdown was lactate rather than glucose. The failure to demonstrate a glucagon response *in vitro* in Patient 6 is disturbing and, so far, unexplained. However, this child's clinical behavior and *in vivo* response to glucagon was similar to that of Patient 1, where an effect on liver slices was demonstrated.

Normally, the blood lactate level is low in the fasting state and rises after a carbohydrate load (22). The fasting venous lactate concentration is higher than the arterial concentration, as muscle is a principal source of this metabolite, while the liver withdraws it from the circulation. In our patients, however, the blood lactate level was highest during fasting and decreased after feeding or glucose infusion. Muscle was not a major source of lactate. This "paradoxical" behavior is readily explained by the hypothesis that hepatic glycogenolysis was the principal source of circulating lactate in these cases. In the fasting state, hypoglycemia developed, hepatic glycogenolysis was stimulated, and the blood lactate level rose. After feeding or glucose infusion, hepatic glycogenolysis ceased, glycogen deposition supervened, and the blood lactate level fell. Glucagon produced sharp increases in blood lactate concentration under all circumstances by inducing rapid hepatic glycogenolysis.

Our conclusion that glucagon may induce hepatic glycogenolysis in some children with liver glycogen disease, just as it does in normal children, is not really in conflict with the findings of other investigators. A glucagon effect was looked for as early as 1932 by van Creveld (23), who administered an intravenous injection of insulin containing the "hyperglycemic factor" to a child with the disease. However, van Creveld examined only the response of the blood glucose level to such injection. Later investigators similarly failed to consider the possibility that some other metabolite might be the end-product of glycogenolysis. However, our observation of a decrease in liver size during glucagon administration may not be unique. Gitzelmann (24) observed a reduction in liver size, comparable with that reported here, in a child thought to have a mild form of liver glycogen disease. He administered smaller doses over a longer period of time, and avoided the side effects we observed with intravenous infusions.

Most reports on liver glycogen disease do not include data on blood lactate levels. However, some authors have recorded strikingly increased fasting blood lactate values in this disorder (21, 25, 26). Mason and Sly (25) observed, as we have, that the blood lactate level was markedly elevated during periods of prolonged hypoglycemia and that it fell toward normal values when the blood glucose level was maintained by frequent administration of glucose. Mason and Andersen (21) presented evidence that the liver, rather than muscle, was the source of the excess lactate during fasting. Excessive production of lactic acid may be largely responsible for the fasting acidosis in some cases of this disease (21, 26).

Confirmation of the points made above has recently appeared in an abstract by Howell, Ashton and Wyngaarden (27). These authors studied three siblings with liver glycogen disease, one of whom was subjected to liver biopsy and found to have a deficiency of hepatic glucose-6-phosphatase. All had high fasting blood lactate levels, comparable with those reported here. Glucagon infusions resulted in rapid and striking rises of blood lactate, but only slow increases in blood glucose. Liver

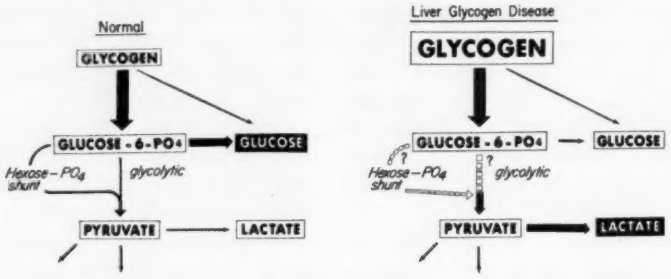

FIG. 5. SCHEMATIC REPRESENTATION OF LIVER GLYCOGEN BREAKDOWN IN PATIENTS 1–6, AS COMPARED WITH NORMAL INDIVIDUALS. Normally, glucose is the principal product of hepatic glycogenolysis. In these patients, lactic acid was the principal product.

slices incubated with glucagon showed appreciable glycogen breakdown and lactate production. The lactate concentration was higher in peripheral arterial than in venous blood, indicating that muscle was withdrawing, rather than contributing lactate to the circulation.

The phenomena discussed above are not seen in all cases of liver glycogen disease, however. Lindsay, Ross and Wigglesworth (28) reported a child whose fasting blood lactate level was only 25 mg per 100 ml. In two cases, van Creveld (23) performed blood lactate determinations; his report does not give the actual values but it may be assumed from the context that they were not remarkable. In one proven case of liver glycogen disease with glucose-6-phosphatase deficiency, recently brought under study, we have found a normal fasting blood lactate level, which did not rise after glucagon administration. No decrease in liver size was observed when this child was given a glucagon infusion. Thus, the findings described in this paper apply only to certain cases of this puzzling disorder.

Our explanation of the hyperlactacidemia in some cases of liver glycogen disease, and its amelioration by glucose, postulates spontaneous hepatic glycogenolysis in response to fasting hypoglycemia and shift toward glycogen deposition when the blood glucose level is raised—i.e., metabolic turnover of liver glycogen. This view was presented earlier by Mason and Sly. This is contradictory to the concept of storage of metabolically unavailable liver glycogen expressed by some authors and implied in the term "glycogen storage disease," used by many. On critical examination, however, the evidence against active metabolic turnover of glycogen in this disease proves to be largely inferential. Many observers have assumed that failure of the blood glucose to rise after administration of glucagon or epinephrine indicates that glycogenolysis did not occur. Such an assumption may be quite erroneous even when the pathways of glycogen breakdown are normal (29) and would certainly be erroneous if the end-product of glycogenolysis is lactate. The fact that glycogenolysis could occur in this disease, either through the glycolytic pathway or through the phosphogluconate pathway, was emphasized by Cori and Cori (30).

In the six patients forming the subject of this report, lactate replaced glucose as the major *in vivo* end-product of hepatic glycogenolysis. Assuming that as in the normal, glucose-6-phosphate is the intermediate from which the metabolic pathways diverge, the sequence of events in these cases might be represented as in Figure 5. The initial stage of glycogen breakdown proceeds much as in normal. Deviations from the normal occur in the metabolism of glucose-6-phosphate and subsequent compounds. No data are available on the relative importance of the hexose monophosphate shunt versus the glycolytic pathway; these are represented by interrupted lines. This scheme postulates quantitative, rather than qualitative, differences from the normal. However, our data do not rule out the possibility that aberrant metabolic pathways may exist in liver glycogen disease; nor

do they define the basic etiologic mechanism for the various abnormalities in this disorder.

SUMMARY

Six children with liver glycogen disease had strikingly elevated fasting blood lactate levels. In all five so studied, the blood lactate concentration fell when hypoglycemia was corrected by feeding or glucose infusions. Rapid return to markedly abnormal blood lactate values was observed after hypoglycemia was allowed to recur. In all six children, glucagon injection resulted in little or no rise in blood glucose, but in prompt and large increases of blood lactate levels. Intravenous infusions of glucagon produced acute decreases of liver size in all the five patients so treated.

Glucagon stimulated glycogenolysis during *in vitro* incubation of liver slices obtained from one of these patients, but only traces of glucose appeared in the medium.

It is concluded that active breakdown of liver glycogen occurred in these patients in response to the stimulus of hypoglycemia, and that glucagon exerted its usual hepatic glycogenolytic effect. However, the principal end-product of liver glycogen breakdown, whether spontaneous or glucagon-induced, was lactic acid rather than glucose.

ACKNOWLEDGMENT

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INTERPRETATION OF CHANGES IN "CENTRAL" BLOOD VOLUME AND SLOPE VOLUME DURING EXERCISE IN MAN *

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Under certain circumstances, the volume of a vascular compartment may be estimated by the indicator dilution technic (1, 2). The method usually has been applied to measure the "central" blood volume (CBV). For this, injections of indicator are made into the right side of the heart, with sampling from a peripheral artery. The blood volume thereby measured comprises that of the heart and lungs and that of an ill defined systemic component extending out to a boundary in all vessels temporally equidistant with the sampling site. The latter component accounts for a large proportion of the total CBV under resting conditions, its precise contribution depending upon the sampling site chosen.

Many recent studies (3–8) have shown an increase in the CBV during exercise in man, its extent depending upon the severity of the exercise undertaken and the site of both injection and sampling. However, it has been pointed out by Lammerant (9) and by Gleason, Bacos, Miller and McIntosh (10) that the proportion of the systemic component to the total CBV may change with redistribution of systemic flow.

The present studies were undertaken to determine whether this commonly reported increase in the CBV during exercise could be explained by an increase in the volume of its vague systemic component. If this were so, conclusions could not be drawn about changes in the volume of blood in the lungs and heart.

METHODS

The subjects were 7 healthy men, aged 25 to 35 years, and 1 woman aged 50 years. Studies were performed after a light breakfast. All of the men were thoroughly familiar with the procedure.

The first series of observations was made on 4 subjects lying supine, standing, and then exercising with varying grades of severity on a treadmill inclined at an angle of 12° from the horizontal. The cardiac output was measured by the indicator dilution method. A no. 5 Lehman cardiac catheter was introduced into a brachial vein by percutaneous puncture and advanced until its tip lay at the lower end of the superior vena cava. The catheter was kept filled with cardio-green dye,[1] and successive injections of 7.5 mg of the dye were made by displacement. The duration of each injection (0.5 to 1.0 second) was signaled on the record. The right arm was kept at rest throughout the procedure. Blood was sampled from the right radial artery at a rate of 30 to 50 ml per minute through a Wood cuvet densitometer and was reinfused after each curve. Samples of blood were taken before and after each complete study; known amounts of dye were added to 10-ml aliquots and drawn through the densitometer to obtain calibrations for the dilution curves. At any given concentration of dye, the deflection obtained with the latter blood sample was 0 to 6 per cent less than that with the former because of the persistence of small amounts of background dye in the blood. Allowance for these minor changes in calibration were made in calculating the area subtended by each indicator dilution curve.

The mean transit time of the recorded dilution curve was corrected for delay owing to the sampling system as follows. By means of the apparatus described by Fox, Sutterer and Wood (11), undyed blood followed by a square wave front of dyed blood was drawn through the sampling system at a rate similar to that used in the experiments. The first derivative of the resulting time-concentration curve was identical in contour with that which would have been obtained had the sampling system been tested with a sudden single injection of dye (11, 12). The mean transit time of this curve was calculated; subtraction from that of the recorded curve gave the corrected mean transit time between injection and sampling sites. The CBV was calculated as the product of cardiac output (milliliters per second) and the "true" mean transit time (seconds).

In other studies, the cardiac output and the CBV were measured during leg exercise in the supine position. In two subjects a blood pressure cuff was applied to the right arm; this was inflated to a pressure of 250 mm Hg for 3 minutes while the subject performed leg ex-

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[1] Kindly supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md.

ercise. The pressure then was suddenly released from the cuff and, while the subject continued to exercise, an indicator dilution curve was obtained from the right radial artery, being completed within 30 seconds of the release of occlusion. It was thus possible to compare the CBV obtained under these circumstances with that obtained while the subject was performing leg exercise of identical severity in the absence of any interference with blood flow to the sampling limb. During this series of observations, blood flow in the forearm was measured by a

TABLE I

Cardiac output, mean transit time and "central" blood volume at rest supine, at rest standing, and during graded exercise on an inclined treadmill *

Subject	BSA	Observation	Condition	Cardiac output	Cardiac index	Mean transit time	"Central" blood volume	
	m^2	no.		L/min	L	sec	ml	ml/m^2
R.M.	2.10	1	Rest, supine	6.90	3.30	18.85	2,160	1,030
			Rest, standing	5.70	2.70	15.90	1,510	720
			1.7 mph	14.30	6.80	12.50	2,980	1,420
			2.5 mph	20.70	9.85	11.20	3,860	1,840
			3.5 mph	25.60	12.20	12.25	5,230	2,490
			Standing after exercise	5.20	2.50	14.05	1,210	580
		2	Rest, supine	6.45	3.10	20.00	2,150	1,020
			Marking time	9.25	4.40	13.50	2,080	990
			3.5 mph	20.00	9.50	14.10	4,700	2,240
		3	Rest, supine	7.35	3.50	17.15	2,100	1,000
			Rest, standing	5.80	2.75	17.50	1,690	800
			Marking time, 1	8.00	3.80	15.15	2,020	960
			Marking time, 2	12.20	5.80	12.30	2,500	1,190
			2.5 mph	15.50	7.40	11.35	2,940	1,400
			3.5 mph	20.00	9.50	11.50	3,830	1,820
			4.5 mph	22.30	10.60	12.45	4,630	2,200
D.W.	1.91	1	Rest supine	7.15	3.75	15.50	1,850	970
			Rest, standing	4.55	2.40	15.75	1,200	630
			1.7 mph	14.20	7.40	8.60	2,040	1,060
			2.5 mph	17.55	9.20	7.15	2,090	1,090
			3.5 mph	20.30	10.60	6.95	2,350	1,230
		2	Rest, supine	6.70	3.50	15.15	1,690	880
			Rest, standing	4.10	2.15	18.35	1,250	650
			1.7 mph	11.30	5.90	9.60	1,810	940
			2.5 mph	16.70	8.75	8.15	2,270	1,190
			3.5 mph	16.60	8.70	8.30	2,300	1,200
		3	Rest, supine	7.50	3.90	14.95	1,870	980
			Rest, standing	3.95	2.05	15.35	1,010	530
			Marking time, 1	6.10	3.20	13.70	1,390	730
			Marking time, 2	7.15	3.75	11.05	1,320	690
			1.7 mph	16.10	8.40	9.20	2,470	1,290
			2.5 mph	17.25	9.05	8.35	2,400	1,260
			3.5 mph	21.60	11.30	6.70	2,410	1,260
Y.W.	1.85	1	Rest, supine	6.55	3.55	19.15	2,090	1,130
			Rest, standing	5.70	3.10	16.10	1,530	830
			1.7 mph	11.10	6.00	13.40	2,480	1,340
			2.5 mph	12.70	6.85	11.20	2,380	1,280
			3.5 mph	18.75	10.15	10.70	3,350	1,810
			4.5 mph	20.20	10.90	9.00	3,030	1,640
			Standing after exercise, 1	5.30	2.85	15.50	1,370	740
			Standing after exercise, 2	4.50	2.45	15.55	1,170	630
		2	Rest, supine	6.05	3.25	23.15	2,340	1,260
			Rest, standing	4.60	2.50	22.50	1,730	930
			Marking time	7.35	3.95	18.50	2,270	1,220
			3.5 mph	19.20	10.35	11.70	3,740	2,020
D.H.	1.73	1	Rest, supine	6.20	3.60	17.05	1,760	1,020
			Rest, standing	4.60	2.65	15.60	1,200	690
			1.7 mph	8.80	5.10	11.90	1,750	1,010
			2.5 mph	12.50	7.20	8.35	1,680	970
			3.5 mph	15.30	8.85	9.45	2,410	1,390
			4.5 mph	14.90	8.60	9.50	2,360	1,360

* Nine observations in four subjects.

modified Whitney mercury-in-rubber strain gage plethysmograph (13, 14) both with the arm at rest and immediately after release of arterial occlusion. In a third subject (J.S.), needles were inserted into both radial arteries, and reactive hyperemia was induced alternately in the two limbs. A fourth subject (W.M.) was kept cold during the early part of the study, and pronounced vasoconstriction was present in the hands and arms. Dilution curves were obtained at rest and during strenuous leg exercise in the supine position. The subject then was warmed by means of blankets and lamps while he continued to exercise at intervals. One hour later, when the hands and arms were warm and he was sweating profusely, dilution curves were obtained during further periods of strenuous exercise, both under normal circumstances and after sudden release of arterial occlusion to the sampling limb.

RESULTS

Observations during upright exercise. Indicator dilution curves from Subject R.M., obtained while resting supine, standing, and then walking up the inclined treadmill at 1.7 and 3.5 miles per hour, are shown in Figure 1. Comprehensive results from all nine studies in the four subjects who performed exercise in the upright position are shown in Table I. In the supine position at rest, the cardiac index averaged 3.54 L, the mean transit time 17.62 seconds, and the CBV 1,034 ml

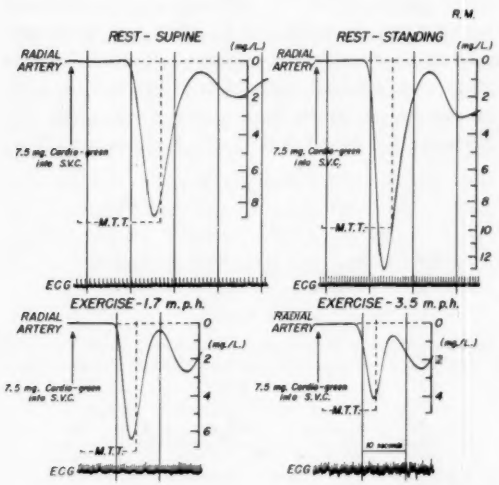

FIG. 1. INDICATOR DILUTION CURVES OBTAINED IN R.M. AT REST AND DURING EXERCISE. The calibrations are on the right. The vertical lines with arrow heads indicate the time of injection. The interrupted horizontal lines show the duration of the mean transit time, corrected for delay in the sampling system.

TABLE II

Mean values and standard deviations for cardiac index, mean transit time and "central" blood volume *

Condition	Type of value	Cardiac index	Mean transit time	"Central" blood volume
		L	*sec*	*ml/m²*
Rest, supine	Mean	3.54	17.62	1,034
	SD	0.20	2.73	185
Rest, standing	Mean	2.54	17.13	722
	SD	0.33	2.33	127
Exercise (3.5 mph on treadmill inclined at 12°)	Mean	10.21	9.69	1,652
	SD	1.15	2.20	457

* Eight observations in four subjects. Studies while standing were not made during the second observation in Subject R.M.

per m² of body surface (Table II). During quiet standing, the cardiac index was reduced to 2.54 L; the mean transit time also was slightly reduced to 17.13 seconds, with a consequent decrease in the CBV to 722 ml per m². During strenuous exercise, the cardiac index was 10.21 L, which was a fourfold increase over the value obtained during quiet standing; the mean transit time was reduced to 9.69 seconds, and the CBV was increased to 1,652 ml per m², an increase by 129 per cent over the value obtained during quiet standing.

The large values obtained for the standard deviation in the measurements of the mean transit time and the CBV are caused by the fact that Subjects R.M. and Y.W. showed considerably less reduction of the mean transit time during strenuous exercise than did D.W. and D.H. This difference appears to be a real one between individuals, since similar values were obtained in successive observations on the same subject. This is illustrated in Figures 2 and 3, in which results from the three separate studies on R.M. and D.W. are shown. Figure 2 shows the line along which points would lie if the calculated CBV were constant, irrespective of posture or exercise. Points falling to the left of and below the line indicate a decrease in the CBV, and points to the right of and above the line indicate an increase. In R.M., at outputs of 12 L per minute and greater, no further significant reduction of the mean transit time occurred, whereas D.W. showed a continual decrease in the mean transit time up to the maximal exercise undertaken. Figure 3 demonstrates for each of the two subjects a substantial decrease in the CBV with assumption of the erect position. However, the increase in the calculated CBV dur-

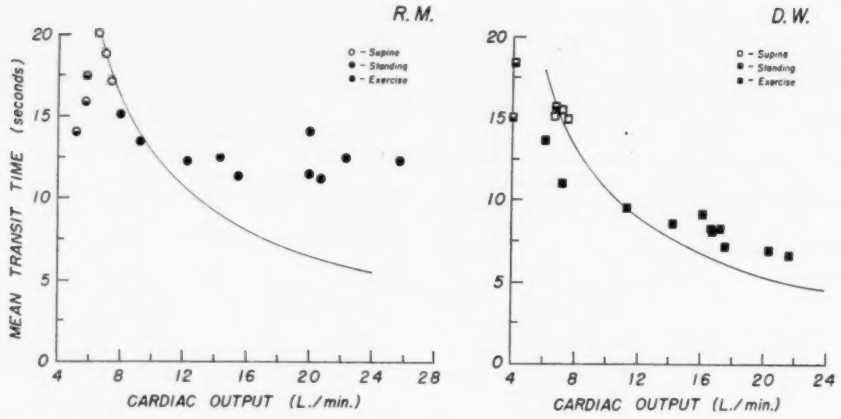

FIG. 2. RELATIONSHIP BETWEEN CARDIAC OUTPUT AND MEAN TRANSIT TIME AT REST AND DURING EXERCISE IN R.M. (LEFT PANEL) AND D.W. (RIGHT PANEL). The continuous lines are those on which the points would lie if the "central" blood volume remained the same during standing and during exercise as that at rest in a supine position. Points to the left of and below the lines correspond to decreases in "central" blood volume, and points to the right of and above the lines correspond to increases.

ing exercise in R.M. was much greater than in D.W.

The CBV was measured in two subjects while they were standing still within 15 minutes of the end of a series of strenuous exercises on the treadmill. Subject Y.W. showed persistent tachycardia, a decrease in arterial blood pressure to 90 mm Hg systolic and 60 diastolic, and facial pallor. The cardiac output in successive determinations was 5.30 and 4.50 L per minute, the stroke volume 41 and 35 ml, and the CBV 1,370 and 1,170 ml, compared with 1,530 ml in the initial determinations with the subject standing prior to exercise. In R.M., the blood pressure was 70/50, the cardiac output 5.2 L per minute, the stroke volume 43 ml and the CBV 1,210 ml, compared with 1,510 ml in the initial observation.

Observations during supine leg exercise. Figure 4 shows indicator dilution curves obtained from the female subject G.J., while she performed mild leg exercise in the supine position. The first curve was obtained without any interference with the circulation in the limb used for sampling. At this time, the blood flow in the forearm was 3 ml

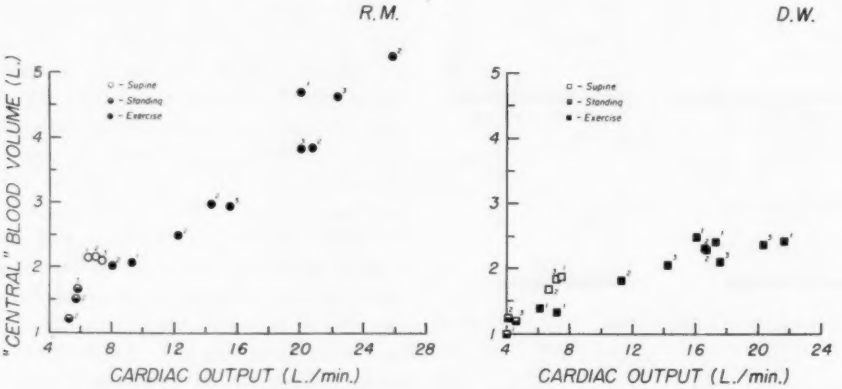

FIG. 3. EFFECT OF CHANGES IN POSTURE AND OF EXERCISE ON "CENTRAL" BLOOD VOLUME IN R.M. (LEFT PANEL) AND D.W. (RIGHT PANEL).

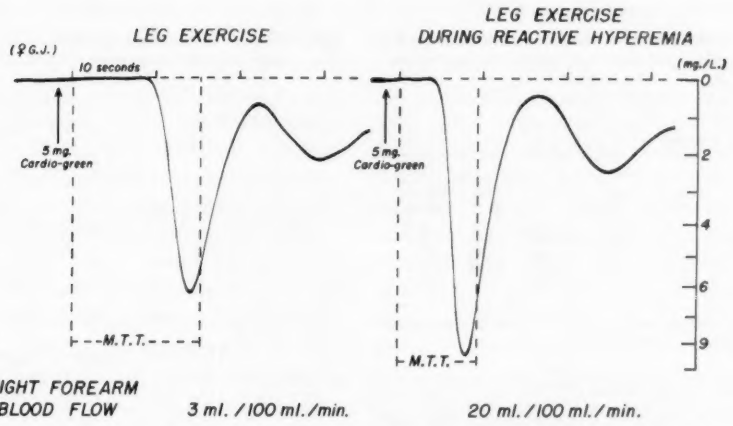

FIG. 4. INDICATOR DILUTION CURVES OBTAINED WHILE G.J. PERFORMED MILD LEG EXERCISE IN THE SUPINE POSITION. The curve in the right panel was inscribed just after release of an arterial occlusion cuff to induce reactive hyperemia in the sampling arm. Note the effect of this maneuver on the contour of the dilution curve, the mean transit time and the forearm blood flow.

per 100 ml of forearm per minute. The second curve was obtained during similar mild leg exercise but immediately after the pressure was released from an arterial occlusion cuff on the sampling limb. At the time of inscription of the dilution curve, the blood flow in the forearm was 20 ml per 100 ml per minute. This resulted in an earlier appearance time, a higher peak concentration, more nearly complete clearing of the dye between the primary circulation and the first recirculation, and a reduction of the mean transit time from 15.3 to 9.2 seconds. The results of this study are summarized in Table III. At rest, the CBV was approximately 1,500 ml; during mild leg exercise, it increased to 2,000 ml, but during similar mild leg exercise, with an increased blood flow to the sampling limb, it was only 1,100 ml, a reduction by about 400 ml from the value obtained at rest.

TABLE III

Effect of reactive hyperemia in sampling arm on measurement of "central" blood volume during supine leg exercise (Subject G.J.)

Condition	Cardiac output	Mean transit time	"Central" blood volume	Forearm blood flow
	L/min	*sec*	*ml*	*ml/100 ml/min*
Rest	4.4	19.9	1,460	3.6
Mild exercise	7.9	15.3	2,000	3.0
Rest	4.8	19.1	1,530	3.4
Mild exercise*	7.5	9.2	1,120	20.0

* Output measured during reactive hyperemia after release of arterial occlusion of the arm used for sampling.

Figure 5 shows indicator dilution curves obtained during strenuous leg exercise in the supine position in J.B. During a period of reactive hyperemia after release of arterial occlusion, the forearm blood flow in the sampling limb was 24 ml per 100 ml per minute, as compared with the control value of 3 ml per 100 ml per minute. The cardiac output corresponding to each of these dilu-

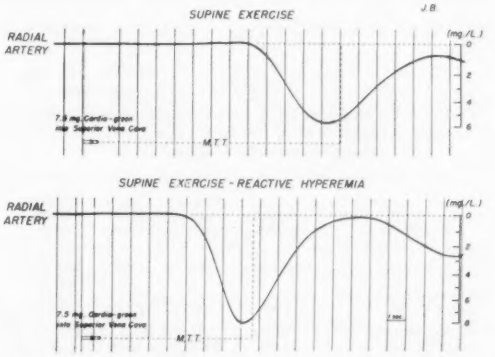

FIG. 5. INDICATOR DILUTION CURVES OBTAINED WHILE J.B. PERFORMED SEVERE LEG EXERCISE IN THE SUPINE POSITION. The curve in the bottom panel was inscribed during a period of reactive hyperemia. Note the reduction of the mean transit time and the increased steepness of the disappearance slope.

TABLE IV

Effect of reactive hyperemia in sampling arm on measurement of "central" blood volume and slope volume during supine leg exercise (Subject J.B.)

Condition	Cardiac output	Mean transit time	"Central" blood volume	Slope volume	Forearm blood flow
	L/min	*sec*	*ml*	*ml*	*ml/100 ml/min*
Rest	7.3	14.2	1,730	280	3.0
Moderate exercise	12.2	14.2	2,890	495	2.8
Moderate exercise*	13.3	8.4	1,860	265	22.6
Severe exercise	15.0	12.5	3,130	590	3.0
Severe exercise*	15.1	7.5	1,890	300	24.0

* Output measured during reactive hyperemia after release of arterial occlusion of the arm used for sampling.

tion curves was approximately 15 L. The pronounced effect of reactive hyperemia in the sampling limb on the contours and time components of the dilution curves is shown in this figure also. The main features are shortening of the appearance time, increased peak concentration, more nearly complete clearance of dye between the primary circulation and the first recirculation, reduction of the mean transit time, and increased steepness of the disappearance slope. The data are summarized in Table IV.

Similar studies were carried out in W.M. (Table V). Initially the subject felt cold; there was severe vasoconstriction of the skin, and the forearm blood flow was 2.2 ml per 100 ml per minute. The calculated CBV was 2,600 ml; during fairly strenuous leg exercise it increased to 3,780 ml. Later, indirect body heating was accomplished by covering the trunk and lower limbs with blankets and using infrared lamps. Identical exercise was

TABLE V

Effect of body heating and of reactive hyperemia in sampling arm on measurement of "central" blood volume during supine leg exercise (Subject W.M.)

Condition	Cardiac output	Mean transit time	"Central" blood volume	Forearm blood flow
	L/min	*sec*	*ml*	*ml/100 ml/min*
Rest, supine, subject cold	7.20	21.65	2,600	2.2
Exercise, supine, subject cold	16.50	13.75	3,780	2.4
Rest, supine, subject cold	7.20	22.25	2,670	2.4
Exercise, supine, subject hot and sweating	16.45	11.20	3,080	7.0
Exercise, supine, subject hot, reactive hyperemia	16.00	8.70	2,320	25.0

TABLE VI

Effect of reactive hyperemia induced alternately in upper limbs on measurement of "central" blood volume (Subject J.S.)

Condition	Cardiac output	"Central" blood volume		Difference in volume between A and B
		A: SVC* to right radial artery	B: SVC* to left radial artery	
	L/min	*ml*	*ml*	*ml*
Rest	7.6	1,440	1,560	−120
Rest, reactive hyperemia in right arm	8.5	1,060	1,530	−470
Rest	7.5	1,300	1,450	−150
Rest, reactive hyperemia in left arm	7.9	1,350	1,000	+350
Exercise, reactive hyperemia in right arm	12.5	1,280	1,800	−520
Exercise, reactive hyperemia in left arm	14.1	1,850	1,400	+450

* SVC = superior vena cava.

performed when the subject was hot and sweating; the forearm blood flow was 7.0 ml per 100 ml per minute and the CBV was 3,080 ml. The exercise was repeated and a dilution curve was obtained just after release of an arterial occlusion cuff, when the blood flow in the forearm was 25.0 ml per 100 ml per minute; the CBV was 2,320 ml, which was less than the value obtained under the original resting conditions.

In J.S. the CBV was determined simultaneously at both radial arteries. Under resting conditions the volumes measured to these two sites were similar. Reactive hyperemia was induced in each arm alternately both at rest and during exercise, the other arm serving as a control. The CBV always was decreased in the hyperemic limb as compared with that in the control limb (Table VI). Under these conditions, changes in the CBV were due entirely to alterations in peripheral blood flow.

DISCUSSION

The results of the studies in which the CBV was measured at rest supine, at rest standing, and during graded exercise on an inclined treadmill are comparable with those reported by other workers. With mild exercise, the CBV equaled or exceeded the value in the resting supine position; it continued to increase with further increase in the severity of the exercise (Table II). At a walking

speed of 3.5 miles per hour, when the oxygen consumption ranged from 1,000 to 1,260 ml per m^2 per minute, the CBV averaged 2.29 times, and the cardiac output 4.02 times, the values in the resting standing position. These values are comparable with those reported by Mitchell, Sproule and Chapman (5), who obtained during strenuous exercise an increase in cardiac output of 4.24 times, and in the CBV of 2.13 times, the values at rest. Roncoroni, Aramendia, González and Taquini (6) studied their subjects standing at rest and performing relatively mild exercise in the upright position. They noted an increase in cardiac output from 3.46 to 7.55 L per minute per m^2 and in the CBV from 940 to 1,520 ml per m^2.

In the studies reported here, the extent of the increase in the CBV during exercise in the upright position varied considerably from one subject to another, although it was relatively constant from study to study in the same subject (Table I and Figure 2). In R.M. it reached a value on one occasion of 5,230 ml, which approached the predicted total blood volume of 6.56 L. Indeed, had a peripheral venous site been employed for injections of indicator, the CBV would have exceeded the total blood volume. Kaufmann (3), who made injections into a brachial vein and recorded dye dilution curves from an ear oximeter, obtained values for the CBV equal to those for total blood volume in two subjects performing relatively mild exercise, the cardiac output being 13.7 and 13.5 L per minute, respectively. However, he did not state how the dilution curves were calibrated.

It is clear from these and other studies (7, 8, 15) that a moderate or pronounced increase occurs in the CBV during exercise. What is the interpretation of this increase? It could be caused by an increase in the volume of blood contained in the heart and lungs, or in that volume situated between the aortic valve proximally and the sampling site and all other temporally equidistant sites distally. Lammerant (9) and Gleason and associates (10), among others, have shown how systemic redistribution of blood flow could alter the geometry of this time-limited volume component, thereby leading to the possibility of drawing erroneous conclusions about changes in the CBV.

The problem could be settled by sampling via an arterial catheter from the root of the aorta after central injections. In the dog it is possible to pass a short, wide-lumen catheter via the carotid artery to the aortic arch and to sample at a rate permitting clearance of the dead space in 0.2 second. This has permitted measurement of the volume of blood in the lungs and left side of the heart (16) at rest and during graded exercise on a treadmill. Further experience has confirmed our earlier finding (17) that this volume increases only slightly from rest to strenuous exercise. Figure 6 shows data obtained from 10 intact dogs. Over the range of activity at which the studies were made, the mean increase in cardiac output was about 200 per cent; however, the lung and left heart volume increased by only 7 per cent. In contrast, when blood was sampled from a peripheral artery during similar exercise, a consistent major increase in the blood volume occurred. This also suggested that the increase in the CBV demonstrated in humans should be sought in the systemic rather than in the cardiopulmonary component of the volume. Evidence that this is so was obtained from our studies in which the velocity of blood flow in the arm used for sampling was increased.

In the first of these subjects (G.J.), the CBV was approximately 1,500 ml while she rested in the supine position. During mild leg exercise, it increased to 2,000 ml. During a second episode of comparable exercise, reactive hyperemia was in-

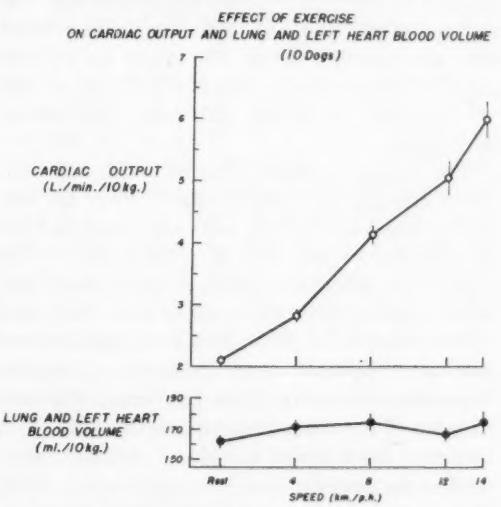

FIG. 6. CARDIAC OUTPUT (TOP PANEL) AND LUNG AND LEFT HEART BLOOD VOLUME (BOTTOM PANEL) IN 10 DOGS STANDING AT REST AND PERFORMING GRADED EXERCISE ON A TREADMILL.

duced in the sampling limb, and the CBV was approximately 1,100 ml, or 400 ml less than the initial value at rest. Since it is unlikely that the deflation of a peripheral blood pressure cuff could have altered the volume of blood in the heart and lungs, this reduction of 900 ml must have been caused by alterations in the systemic arterial component of the volume. During the first episode of leg exercise, the velocity of blood flow to the lower half of the body was increased relative to that to the right arm; during the second period of exercise, the blood flow to the right arm was increased sevenfold, and the time taken for blood to reach the sampling site was reduced by 6 seconds. Hence, the velocity of blood flow down the sampling limb now exceeded that to the lower half of the body. Thus, the former circumstance was characterized by an expansion and the latter by a contraction in the time-equivalent boundaries of the volume.

Similar results were obtained in the other three subjects in whom the effects of induced changes in the velocity of blood flow in the sampling limb were studied. In W.M., body heating increased the forearm blood flow from 2.4 to 7.0 ml per 100 ml per minute, and this resulted in a decrease from 3,780 to 3,080 ml in the CBV during successive periods of comparable leg exercise. In a third episode of exercise, reactive hyperemia also was induced in the sampling limb; the forearm blood flow was now 25.0 ml per 100 ml per minute, and the CBV was reduced further to 2,320 ml, or 300 ml less than the initial value when the subject was at rest.

The remote possibility that the deflation of a blood pressure cuff might reflexly affect the volume of blood in the heart and lungs was ruled out by the observations in J.S. (Table VI). The CBV was measured simultaneously from both radial arteries. The observations were made first with the circulation through each arm undisturbed and were repeated after induction of reactive hyperemia alternately in the two arms. On each occasion, the volume measured to the limb with increased blood flow was reduced and that measured to the opposite limb was unchanged. Thus, the reduction in the former volume was caused by an alteration in its time-equivalent systemic boundaries and not by any change in the cardiopulmonary volume.

Our results confirm and extend the observations of Gleason and associates (10), who sampled simultaneously from the brachial and femoral arteries in resting subjects before and after the release of arterial occlusion cuffs applied to the legs. This maneuver was accompanied by a consistent decrease in the calculated CBV in samples from the femoral artery and by a slight increase in samples from the brachial artery. They concluded that alterations in the CBV cannot be attributed to changes in its intrathoracic component unless it can be shown that any change in blood flow occurs uniformly throughout the arterial system, a most unlikely circumstance.

Braunwald and Kelly (8) noted a slight decrease in the volume between the superior vena cava and the brachial artery when the sampling limb was heated. During leg exercise, the calculated CBV to the warmed limb was greater than under control conditions at rest. However, heating the limb causes only a relatively slight increase in local blood flow compared with that during reactive hyperemia (Table V) and especially with that during strenuous leg exercise (18). Hence, their findings are still fully consistent with the interpretation that the demonstrated increase in the CBV during leg exercise was largely or wholly in the "arterial" component.

The conclusions of many previous studies on patients with heart disease have been based upon the assumption that changes in CBV indicate changes in the volume of blood inside the thorax (19–22). Since an extensive redistribution of systemic venous and arterial blood flow and blood volume occurs in patients with heart disease, such conclusions are invalid. Views that the pulmonary blood volume may act as an important blood reservoir regulating the cardiac output (23–25) are based upon studies to which similar criticism applies. Johnson (23), Lee, Churchill-Davidson, Miles and de Wardener (26), and Etsten and Li (27) noted a decrease in CBV during general anesthesia. However, since the forearm blood flow during anesthesia increases despite a decrease in cardiac output (26), a reduction in the arterial component of the CBV would be expected. This, rather than a change in pulmonary blood volume, could explain these findings. The increase in CBV during infusion of trimethaphan (Arfonad) camphorsulfonate, a ganglion-blocking drug (28),

also could be related to its effects on the peripheral rather than the pulmonary circulation.

Thompson, Berry and McIntosh (7) measured the CBV in patients who were hyperventilating and also while they were performing leg exercise. Each procedure doubled the cardiac output. The CBV increased with leg exercise but not with hyperventilation, and the conclusion was that the response of the cardiac output to these two maneuvers may be brought about by different mechanisms. An alternative explanation is that during hyperventilation, as opposed to leg exercise, the velocity of blood flow to the lower part of the body is unlikely to increase relative to that through the sampling limb, and hence an increase in the systemic arterial component of the volume would not be expected.

In eight observations on four of our subjects, a mean decrease of 30 per cent occurred in the CBV on changing from the supine to the standing position. This is similar to the decrease of 25 per cent noted by Weissler, Leonard and Warren (25). It is indeed probable that the cardiopulmonary component of the volume does diminish but, since qualitative changes in systemic flow occur with the change in posture, it cannot be determined how much of the decrease should be attributed to the lungs and how much to changes in the "arterial" volume. In the absence of more precise data, statements such as "on standing from the supine position about one-quarter of the blood in the lungs goes into the legs" (29) are best avoided.

Thus it is clear that, when either the injection site or the sampling site is peripheral, any maneuver that causes redistribution of systemic blood flow, such as exercise, change of posture or general anesthesia, will be associated with a change in the calculated CBV. Attempts to equate this change quantitatively or even qualitatively with a change in the volume of blood in the lung vessels are, however, fraught with hazard.

Much of the confusion has arisen from attempts to think of the CBV as a conventional three-dimensional volume. Its limits, however, do not conform to any fixed anatomic boundary but are determined by equivalence in a fourth dimension, namely time. The elusive nature of such a volume is attested to by the number of synonyms that have been used, including the intrathoracic volume, the cardiopulmonary volume and the needle-to-needle volume, none of which is satisfactory. From these considerations, it is difficult to justify any further studies on such a nebulous entity as the CBV as it usually has been measured.

McGaff, Jose and Milnor (30), and Dock and co-workers (31) recently attempted to measure the pulmonary blood volume in man by making successive or simultaneous injections into the pulmonary artery, sampling from a common peripheral site, and subtracting the volumes calculated from the corresponding dilution curves. This technic avoids fallacies from changes in the systemic component of the volume. However, the left atrium may be a poor mixing chamber, particularly in patients with rheumatic heart disease. Sampling from the aortic root after injections into the pulmonary artery also avoids fallacies and fulfills the criteria for good mixing at both injection and sampling sites (32). This method has been used in animals (17), but it also has a disadvantage in that the combined blood volume of the lungs and left heart chambers is measured.

Newman, Pearce, and associates (33, 34), developed the concept that the "slope volume," which is calculated from the product of the cardiac output and the rate of declining concentration of indicator in the disappearance phase of the dilution curve, is related to a pulmonary mixing volume. Recently, a more guarded definition of the slope volume has been used (35). It appeared possible that calculation of the slope volume might aid in the interpretation of changes in the pulmonary blood volume occurring during exercise. However, it became clear that the slope volume, like the CBV, was greatly influenced by the systemic redistribution of blood flow. In Subject J.B. (Table IV), the slope volume during strenuous leg exercise was 590 ml. During an identical episode of exercise, reactive hyperemia was induced in the sampling arm. The cardiac output was unchanged, but the disappearance slope was twice as steep; thus, the slope volume was reduced to 300 ml. This, together with similar although less pronounced findings in other subjects, is evidence that the slope volume is affected by linear dispersal of the indicator in peripheral vessels, supporting our recent conclusion from animal studies (36) that the slope volume does not reflect changes in the pulmonary blood volume either quantitatively or qualitatively.

SUMMARY

The "central" blood volume (CBV) was measured in four subjects at rest supine, standing, and during exercise on an inclined treadmill. Injections of indicator were made into the superior vena cava, and dilution curves were obtained from the radial artery. Mean values for the CBV were 1,034 ml per m^2 at rest supine, 722 ml per m^2 while standing, and 1,652 ml per m^2 during walking at 3.5 miles per hour.

In four further subjects, the CBV was increased during the performance of leg exercise in the supine position. Such exercise is associated with an increased flow of blood to the lower limbs. When the rate of blood flow to the arm used for sampling also was increased by sudden release of pressure from an arterial occlusion cuff, a great decrease in the CBV occurred, the values approaching, or even falling below, those obtained under the initial resting conditions. Thus, the increase in the CBV during exercise is partly or wholly caused by an increase in its systemic "arterial" component, in consequence of relative changes in the rates of blood flow to different regions.

Failure to appreciate the significance of such hemodynamic changes, during exercise, change of posture and general anesthesia, has led in the past to unwarranted conclusions about changes in pulmonary blood volume, the reservoir function of the lungs, and the interdependence of pulmonary blood volume and cardiac output. It is difficult to see how any valid information about the pulmonary blood volume can be obtained from measurement of the CBV when a peripheral sampling site is used.

Evidence has been obtained that the "slope volume" is influenced by linear dispersal of indicator in peripheral arteries. Thus, this volume no longer can be held to reflect either quantitatively or qualitatively the volume of any single component of the central circulation, such as the lungs.

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THE RELATION BETWEEN EXTERNAL POTASSIUM CONCENTRATION AND THE ELECTROLYTE CONTENT OF ISOLATED RAT MUSCLE IN THE STEADY STATE *

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There have been many studies of electrolyte fluxes in isolated mammalian muscle (1–4). However, there exists at present virtually no information about the factors which *in vitro* affect the steady state concentrations of intracellular electrolytes. To investigate this problem a technique for the incubation of intact rat diaphragms has been developed which permits the prolonged maintenance of muscle composition in a steady state.

The purpose of this communication is, first, to describe the technique and to present evidence that a stable and physiological tissue composition can be maintained for up to 12 hours. Second, data are presented which define the effects of varying external concentrations of potassium on muscle composition. Striking and unexpected differences between *in vivo* and *in vitro* behavior were encountered.

METHODS

Sprague-Dawley rats, 40 to 80 g in weight, were lightly anesthetized with intraperitoneal 4 per cent sodium amobarbital solution. The entire thoracic rib cage was dissected out, leaving the diaphragm intact. Special care was taken not to cut the diaphragm or its attachments. The diaphragm with the rib cage attached was then rinsed quickly in saline before being placed in the bathing medium.

The bathing solution was a modified Krebs-Ringer bicarbonate medium containing: NaCl, 115 mmoles per L; $NaHCO_3$, 25 mmoles per L; NaH_2PO_4, 1.2 mmoles per L; $MgSO_4$, 1.2 mmoles per L; and $CaCl_2$, 1.3 mmoles per L. KCl was added as dictated by experimental design, the Na content remaining constant. Glucose, 100 mg per 100 ml was added in every case. For antibacterial effect, chloramphenicol, 62.5 mg per L was used, sometimes supplemented with penicillin, 100,000 U per L, and streptomycin, 100 mg per L.

The bathing solution was contained in a specially designed plastic box with a capacity of approximately 3 L. The box had a sintered glass bottom plate which permitted vigorous aeration of the medium with 95 per cent O_2 and 5 per cent CO_2. The incubating box was kept in a constant temperature water bath which maintained the fluid in the box at 37° C. The pH of the bathing solution, checked at intervals, was kept within the range of 7.37 and 7.43.

At the beginning of an experiment 12 diaphragm preparations were placed in 1,500 ml of bathing medium within the plastic box and secured to glass rods in order to keep them fully immersed. During the first hour of an experiment approximately 500 ml of bathing solution was changed every 15 minutes. Thereafter 500 ml was changed every 30 minutes until the end of the experiment.

At the end of an experiment the intact diaphragm preparations were washed in 5 per cent glucose solution and the two lateral muscular hemidiaphragms were rapidly dissected from their rib cage attachments and central tendons. The hemidiaphragms were then blotted once on filter paper, placed in tared test tubes and weighed. In rats of the size used in these experiments the muscular portion of the diaphragm is less than 0.5 mm in thickness (4 to 8 muscle fibers) and its total weight is 100 mg or less. Thereafter the diaphragms were dried under vacuum at room temperature for 15 hours and reweighed to obtain dry weights. Muscle electrolytes were extracted in boiling water for 1 hour, a technique previously validated by comparisons with standard dry-ashing methods. Sodium and potassium analyses of the extract were made with a flame photometer and chloride analyses amperometrically with an Aminco-Cotlove chloride titrator. Diaphragms from *in vivo* control rats were extracted and analyzed in a similar fashion.

Inulin space was used as a measure of the extracellular space of tissue. At least 60 minutes before the diaphragm preparations were to be removed for analysis,

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they were transferred to a second bathing solution, identical with the first except for the presence of inulin, approximately 700 to 900 mg per 100 ml. In agreement with the observations of Creese (5), it had previously been established that a period of 60 minutes was more than sufficient time to permit equilibration of the inulin.

The alkali-stable fraction of inulin in the tissue extracts was prepared for analysis by a modification[1] of Cotlove's technique (6). Five ml of extract was placed in 10-ml volumetric flasks, 1.6 ml of 0.75 N NaOH added, and the solutions heated in a water bath for 30 minutes. The pH was then adjusted with 1.5 N HCl to approximately 7.0, using phenolphthalein as an indicator. Protein was precipitated by adding exactly 1 ml of 10 per cent $ZnSO_4$ and 1 ml of 0.75 N NaOH. The solutions were then made up to 10 ml volume, shaken, allowed to stand for 10 minutes, and centrifuged. Inulin analyses were performed on 2 ml of the supernatant, using the resorcinol method of Roe, Epstein and Goldstein (7). The inulin analysis of the bathing solution was carried out in a similar fashion after appropriate dilution. To determine the *in vivo* inulin space of the diaphragms, bilateral nephrectomies were performed and 1.5 ml of a 10 per cent inulin solution was injected intraperitoneally or intravenously. The animals were exsanguinated after 3 hours and the diaphragms analyzed for inulin as already described. In *in vivo* and *in vitro* experiments an average blank correction for inulin was made by appropriate analyses of groups of diaphragms. Blank values were always less than 5 per cent of the total inulin color.

RESULTS

Stability of the preparation. Table I summarizes the results obtained in one series of experiments in which diaphragms were incubated for periods up to 12 hours at a bath potassium concentration of 6 mEq per L. Total water content, as per cent of wet weight, rose slightly during the course of the experiment. This was mainly due to a small increase in extracellular water, which became apparent at the ninth and twelfth hours. Intracellular water was relatively constant after a slight initial increase. At first, calculated intracellular potassium dropped by 18 mEq per L from *in vivo* control levels and intracellular sodium rose by 29 mEq per L. After these initial changes, intracellular electrolyte composition remained essentially constant throughout the 12 hour period. The sodium entering cell water in excess of the loss of potassium was accompanied by a simultaneous gain of intracellular chloride. The inherent inaccuracy of the calculation of intracellular sodium and chloride prevents any precise comparison between gains in these ions and the loss of potassium.

The stability and physiologic viability of the preparation is emphasized by the results of a group of experiments shown graphically in Figure 1. In these studies the muscles were placed in a potassium-free bath during the initial period of incubation. After 4 hours some of the muscles were taken for analysis and it was found that approximately half the potassium content had been lost in exchange for an equivalent amount of sodium. The rest of the diaphragms were then placed in

[1] The authors are grateful to Dr. Cotlove for suggesting this adaptation of his technique.

TABLE I

Changes in water compartments and electrolyte composition of rat diaphragm during incubation at bath potassium of 6 mEq per L

	Water	Inulin space	Total content			Intracellular water	Intracellular conc.		
			K	Na	Cl		K	Na	Cl
	% wet wt		*mEq/100 g dry wt*			*ml/100 g dry wt*	*mEq/L ICW*		
In vivo control	76±2* [39]†	17±2 [21]	40±2 [40]	12±2 [38]	4±1 [10]	251±20 [21]	158±8 [21]	8±6 [19]	0 [10]
In vitro									
3 hours	77 ± 1 [30]	18 ± 4 [23]	36 ± 2 [29]	20 ± 4 [27]		261 ± 21 [23]	140 ± 10 [22]	37 ± 23 [20]	
6 hours	77 ± 1 [36]	18 ± 4 [29]	37 ± 3 [36]	21 ± 4 [35]		266 ± 19 [29]	139 ± 10 [29]	40 ± 17 [28]	
9 hours	78 ± 1 [30]	21 ± 4 [25]	38 ± 3 [28]	23 ± 6 [27]	14 ± 2 [10]	264 ± 19 [25]	146 ± 14 [24]	41 ± 21 [23]	11 ± 7 [10]
12 hours	79 ± 1 [22]	22 ± 2 [19]	37 ± 2 [22]	21 ± 3 [22]	15 ± 3 [6]	268 ± 15 [19]	137 ± 11 [19]	26 ± 9 [19]	8 ± 6 [6]

* Mean ± standard deviation.
† Number of analyses in brackets.

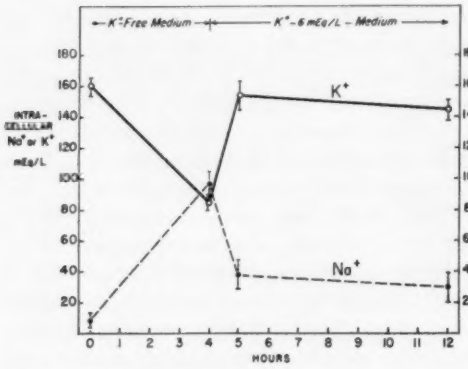

FIG. 1. CHANGES IN INTRACELLULAR POTASSIUM AND SODIUM DURING LEACHING IN A POTASSIUM-FREE BATH AND FOLLOWING THE RESTITUTION OF POTASSIUM TO THE MEDIUM. Each point is the average of 15 to 25 muscle analyses. The vertical lines through each point represent the range of ±2 SE.

a bath containing 6 mEq per L of potassium. In this medium, the muscles rapidly reaccumulated potassium and extruded sodium, as evidenced by the "normal" *in vitro* potassium and sodium content after only 1 hour of incubation. Moreover, these concentrations were maintained in diaphragms incubated for a full 8 hours after leaching, i.e., for a total incubation time of 12 hours.

Relation between bath potassium and muscle potassium content. The time course of muscle potassium content at a variety of bath potassium concentrations is plotted in Figure 2. At a bath potassium of 6 mEq per L muscle potassium was approximately constant throughout the final 9 hours of incubation, as noted above. At a bath potassium of 1.1 mEq per L, muscle potassium fell initially but then rose gradually until it reached the "normal" *in vitro* value for a bath potassium of 6 mEq per L. Potassium content then remained constant during the final 3 hours of incubation. At bath potassiums of less than 1 mEq per L, muscle potassium content in each case fell progressively during the early hours of incubation, but then reached a relatively stable value during the final 4 hours in the bathing medium. The values for this final period, taken to represent an approximately steady state for potassium content, are shown in Figure 2 by the solid points in each line. Each point represents the average of 6 to 12 diaphragms. The mean of all the analyses indicated by the solid points in each line (± its standard

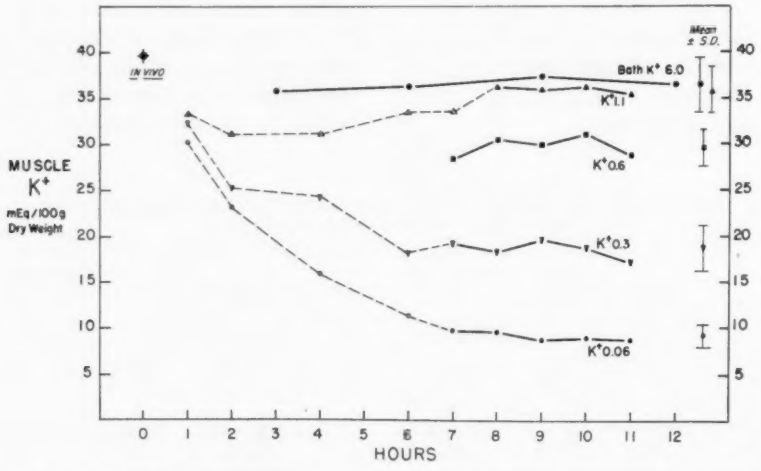

FIG. 2. MUSCLE POTASSIUM CONTENT DURING INCUBATION AT VARIOUS EXTERNAL POTASSIUM CONCENTRATIONS. Each open point connected by broken lines represents the mean of relatively few diaphragms taken early in the experiments to show the general trend of muscle potassium. Each solid point connected by unbroken lines represents the mean of 6 to 12 diaphragms. The mean of all the solid points at each external potassium is represented by the point at the end of the appropriate curve, and the standard deviation of this mean is depicted by the vertical lines through each of these points.

deviation) is plotted at the right of the appropriate curve. These mean potassium contents are each derived from no less than 24 diaphragms taken in at least two separate experiments, in which there was little or no change during the final 4 to 5 hours of incubation. Each mean is considered to approximate a steady state value for muscle potassium content at a given external potassium concentration.

The steady state muscle potassium contents from the experiments depicted in Figure 2, plus comparable values from similar experiments at other bath potassium concentrations, are plotted against external potassium concentration in Figure 3. Three facts about this relationship are worth particular comment. First, there was little or no change in muscle potassium as bath potassium was lowered from 6 to 1 mEq per L, and at most a small increment in muscle potassium as bath potassium was raised to 9.5 mEq per L. At approximately 1 mEq per L there was a sharp break in the curve and, at bath potassiums of less than 1 mEq per L, muscle potassium and bath potassium were linearly related. Because of continuing potassium loss from rib cage structures other than the diaphragm, it was not possible to achieve bath potassium concentrations of less than 0.06 mEq per L. At this concentration (lowest point on curve in Figure 3), some 9 mEq of potassium per 100 g dry weight remained in the diaphragm. Extrapolation of the linear relationship of internal to external potassium from this point to zero external potassium (indicated by the dashed portion of the curve) suggests that some 6 to 7 mEq of potassium per 100 g dry weight would remain in the muscles in a potassium-free bath.

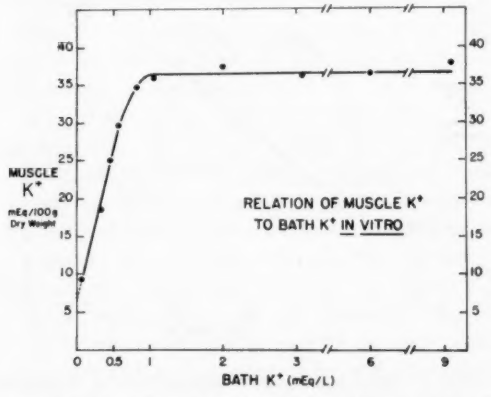

FIG. 3. STEADY STATE DIAPHRAGM POTASSIUM CONTENT *IN VITRO* AS A FUNCTION OF EXTERNAL POTASSIUM CONCENTRATION. Each point represents the mean of 24 to 48 muscles from at least two separate experiments, showing little or no change in muscle potassium content for 3 to 5 hours.

Relation between bath potassium and muscle composition. The upper half of Table II shows the intracellular electrolyte composition of muscles at several external potassium concentrations of 1.1 mEq per L or above. The values given at each bath potassium represent the means of 15 to 22 diaphragms taken after 8 to 10 hours of incubation in several separate experiments. As would be expected from the lack of change in potassium content per dry weight shown in Figure 3, intracellular potassium decreased relatively little as external potassium was lowered from 9.5 to 1.1 mEq per L. Intracellular sodium rose slightly, but chloride was virtually constant. Inulin space and extracellular water were also relatively stable. These results confirm the impression derived from Figure 3 that intracellular electrolyte composition was relatively constant over a wide range of external potassium concentrations greater than 1.1 mEq per L.[2]

Similar data from three experiments at external potassium concentrations of less than 1 mEq per L are summarized in the bottom half of Table II. In each experiment the fall in intracellular potassium concentration from that found at a bath potassium of 1.1 mEq per L was balanced by an approximately equal rise in intracellular sodium. There was little or no change in intracellular chloride. There was no evidence of a significant cation deficit in cells during *in vitro* potassium depletion of muscles, and thus no need to postulate a shift of hydrogen ion or other cations to account for these data.

Differences between the effect of lowered external potassium in vivo and in vitro. Other in-

[2] Note that the values for inulin space and intracellular water and electrolytes were somewhat different at an external potassium of 5.5 mEq per L, in this group of experiments, from those shown in Table I. The two groups of experiments were separated by a year or more. We have no explanation for these differences, in terms of changes in technique, animal size or strain, and so forth; however, results have been quite consistent during the course of the year or more during which the studies shown in Table II were carried out.

TABLE II

Effects of bath potassium on water compartments and electrolyte composition of rat diaphragm

Bath K	Inulin space	Intracellular water	Intracellular electrolytes			
			K	Na	Na+K	Cl
mEq/L	*% wet wt*	*ml/100 g dry wt*	*mEq/L intracellular water*			
9.5 [15]†	26 ± 3*	230 ± 15	167 ± 8	11 ± 5	178 ± 8	4 ± 4
5.5 [18]	25 ± 2	229 ± 11	162 ± 6	15 ± 5	177 ± 7	4 ± 3
1.1 [22]	24 ± 3	239 ± 10	148 ± 8	23 ± 7	172 ± 7	6 ± 3
0.45 [6]	26 ± 1	243 ± 9	116 ± 6	48 ± 7	164 ± 8	4 ± 4
0.29 [5]	25 ± 2	227 ± 6	84 ± 4	93 ± 6	177 ± 4	13 ± 8
0.25 [6]	27 ± 1	222 ± 13	68 ± 7	106 ± 7	174 ± 8	10 ± 5

* Mean ± SD.
† Number of diaphragms in brackets.

vestigators (8) have reported that there is an approximately linear relationship between peripheral skeletal muscle potassium and plasma potassium during potassium depletion *in vivo*. The data presented above indicate that muscle potassium changes but little when external potassium is decreased over the range found in plasma during potassium depletion *in vivo*. To confirm this apparent difference between the *in vivo* and *in vitro* results, two sets of experiments were done. In the first type of experiment, groups of rats similar in size to those used for the *in vitro* experiments were placed on a potassium-free diet for one to three weeks.[3] At intervals, small groups were killed and plasma and diaphragms were analyzed for potassium. The results are shown by the points in Figure 4, each of which represents a single animal. The observed relationship between diaphragm potassium and plasma potassium can be described by a straight line—$y = 4x + 23.5$ (the heavy line in Figure 4); the correlation coefficient (R) is 0.7. These *in vivo* results, showing a linear relationship between diaphragm and plasma potassium, confirm the data of Welt and co-workers (8) obtained with other skeletal muscles. They contrast sharply with the *in vitro* relationship described by the dotted line in Figure 4.

This difference between the *in vitro* and *in vivo* relation of internal to external potassium was demonstrated in another way by the results of a second type of experiment which are summarized in Table III. Thirty-nine rats were placed on a potassium-free diet for several weeks,

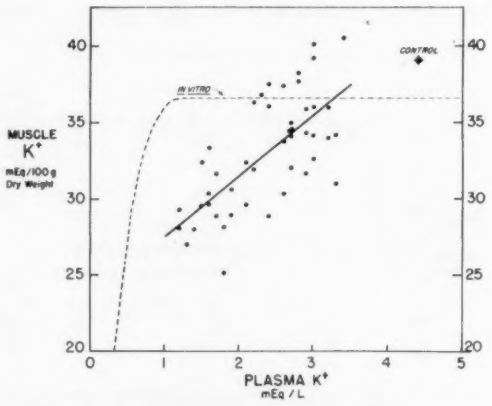

FIG. 4. RELATION OF DIAPHRAGM POTASSIUM TO PLASMA POTASSIUM *IN VIVO*. Each point represents the values from one potassium-depleted rat. The solid line has been fitted to these points by the method of least mean squares. The dashed line is the *in vitro* curve from Figure 3. The point labeled "control" is the mean *in vivo* diaphragm potassium from 51 normal rats.

[3] The diet used was low in alkali, and bicarbonate was not added to the drinking water. Although potassium-deficient, these animals did not become alkalotic, as evidenced by plasma CO_2 values in the same range as those of control rats (25 to 30 mmoles per L).

TABLE III

Effect of incubation on potassium content of diaphragms from potassium-depleted rats

	Plasma K	Bath K	Muscle K
	mEq/L	*mEq/L*	*mEq/100 g dry wt*
In vivo [16]	1.6 ± 0.3		29.5 ± 2.1
In vitro [23]	1.7 ± 0.3	1.1	34.3 ± 3.1

after which 16 of these animals selected at random were killed and their diaphragms taken for analysis. Diaphragms from the remaining 23 rats were incubated for 8 to 10 hours in a bath containing 1.1 mEq per L potassium. The mean plasma potassium concentrations of the two groups were equal, and considerably *higher* than the bath potassium concentration. Nevertheless, the incubated diaphragms actually gained potassium to approximately the "normal" *in vitro* level. Thus muscle potassium content *increased* significantly ($p < 0.001$) when the diaphragms were taken from a higher external potassium *in vivo* to a lower external potassium *in vitro*.

DISCUSSION

An *in vitro* muscle preparation suitable for the study of steady state phenomena should be capable of maintaining its cellular water content and electrolyte concentrations close to *in vivo* levels when incubated in simple physiological media. Cell composition, furthermore, should remain reasonably steady over relatively prolonged periods of time. Rat diaphragm muscle has been widely used for various types of physiological and biochemical studies because it is easily handled and particularly because its thin shape facilitates the rapid equilibration of oxygen and other solutes between muscle and medium. However, cut diaphragm preparations when incubated usually show a progressive loss of potassium and a gain of sodium and water (9, 10). Creese (5) described a cut preparation which appeared capable of maintaining a nearly normal intracellular potassium content for as long as 12 hours, but its intracellular sodium content increased markedly.

Kipnis and Cori (11) pointed out several years ago that an "intact" diaphragm preparation in which the fibers are left attached to ribs and central tendon offers important advantages for the study of normal cellular permeability to sugars. It seemed reasonable to expect that electrolyte equilibrium in such an intact diaphragm would also be more satisfactory, and such has proven to be the case in the present experiments. The technique described here not only permits the maintenance of a nearly physiological cellular sodium, potassium and water content for many hours, but also possesses the additional advantages of simplicity and convenience. Moreover, many diaphragms may be handled simultaneously with relative ease.

The most striking observation made in these experiments was the relative constancy of tissue potassium as external potassium was varied from normal extracellular levels down to very low levels. As shown in Table II, the intracellular concentration of potassium decreased by only 10 per cent when bath potassium was reduced from 5.5 to 1.1 mEq per L, and the total potassium content of the tissue decreased by less than 5 per cent (Figure 3). This contrasts sharply with the steep slope of the line relating external potassium to skeletal muscle potassium content in potassium-depleted animals found by Welt and co-workers in peripheral muscle (8) and confirmed here for diaphragm muscle (Figure 4). *In vivo,* muscle loses 30 per cent or more of its potassium content when plasma potassium is reduced from 4 to 1 mEq per L. When total exchangeable potassium is used as a rough index of tissue potassium content, similar large reductions in tissue potassium with even smaller reductions in plasma potassium can be inferred from observations in potassium-depleted patients (12). Balance studies in two patients with severe chronic potassium depletion (13) indicated a loss of approximately 25 per cent of cellular potassium content with a reduction in plasma potassium to the range of 2.1 to 2.7 mEq per L.

Extracellular acidity is known to affect the *in vivo* relationship between extra- and intracellular potassium, and there may well be other important influences, as yet undefined. However, it is clear from the studies of Welt and co-workers that a reduction in extracellular potassium will lower muscle potassium content in rats with normal extracellular acid-base balance as much as in alkalotic animals. Futhermore, as already noted, the potassium-depleted rats used in the present

in vivo experiments did not become alkalotic. Therefore, the difference between the *in vivo* and *in vitro* behavior of muscle potassium is not to be attributed to the constancy of the pH and bicarbonate content of the incubation medium used in the present studies. The present data make it clear that loss of muscle potassium in potassium depletion is not solely the result of a lowered extracellular concentration of potassium. Other important factors must be involved. Current studies in this laboratory are concerned with this problem.

When the concentration of potassium in the medium was lowered below 1 mEq per L, muscle potassium content began to decline and, as shown in Figure 3, at medium levels below 1 mEq per L there was a direct linear correlation between internal and external potassium. While the data do not rule out a more complex mechanism, it is tempting to speculate that the simple and distinctive shape of the curve in Figure 3 reflects the operation of a single physical process responsible for potassium accumulation. However, the available information does not permit a critical choice among the various mechanisms proposed for this process. Thus, a relationship of this general type might be anticipated if potassium accumulation were entirely the passive consequence of the active extrusion of sodium (2). In that case, as external potassium is lowered, a critical concentration might be reached below which net influx of sodium and consequent loss of potassium might begin. Such an effect might depend on a relationship between external potassium and either sodium pumping or the passive permeability of the membrane to sodium. Alternatively, a curve similar to Figure 3 might also be anticipated if potassium accumulation were the result of active inward transport (14) either as a process independent of sodium efflux, or coupled with it (4). Under either of these latter possibilities, the distinctive shape of the curve could be determined by a saturation phenomenon. A similar explanation could also apply if potassium accumulation were the result of the specific attraction of potassium to fixed negative charges inside the cell (15–18).

Another feature of the data in Table II worth comment is the relationship between loss of intracellular potassium and gain of intracellular sodium. As shown in the column headed "Na + K" the sum of these two ions remained approximately constant as bath potassium was lowered from 9.5 to 0.25 mEq per L, indicating roughly equivalent replacement of potassium by sodium. This is true even if a correction is made for the small and statistically insignificant rise in intracellular chloride at the lowest levels of bath potassium. In most studies of the effects of potassium-depletion on rat muscle *in vivo,* the rise in intracellular sodium is found to be only 70 or 80 per cent of the total loss of intracellular potassium (19–21). Although many workers have assumed that a shift of hydrogen ions into cells makes up this deficit, more recent studies by Eckel, Norris and Pope (21) have shown that in rats the apparently missing cation can sometimes be accounted for by the intracellular accumulation of cationic amino acids, chiefly lysine. No amino acids were added to the bathing medium in the present experiments, and therefore no cationic amino acids could have been available for exchange with intracellular potassium. The data in Table II demonstrate that, despite this, no significant hydrogen-for-potassium exchange occurred.

Finally, it should be noted that extrapolation of the curve in Figure 3 suggests the existence of a significant fraction of tissue potassium which is relatively nondiffusible. This fraction seems nevertheless to be able to exchange freely, since Creese found that K^{42} appeared to equilibrate with total tissue potassium in diaphragms incubated at 38° C (5). It is interesting, however, that at lower temperatures, an apparently nonexchangeable moiety can be demonstrated both in rat diaphragm (22, 23) and in extensor digitorum longus (24). Heterogeneity of intracellular potassium has been suggested by many workers and might conceivably result either from separation into subcellular compartments or from binding of potassium to intracellular constituents.

SUMMARY AND CONCLUSIONS

A method is described for the incubation of intact rat diaphrgams which permits the maintenance of a stable and physiological tissue composition for periods up to 12 hours. A study of the steady state relationship between internal composition and external potassium concentration revealed:

1. Below bath potassium concentrations of 1 mEq per L there was a linear relation between

muscle potassium content and bath concentration. Intracellular potassium was replaced by virtually equivalent amounts of sodium.

2. At a bath potassium of 0.06, about one-fourth of the total tissue potassium remained *in situ* and extrapolation to a potassium-free medium suggests the existence of a significant fraction of intracellular potassium which is relatively non-diffusible.

3. There was essentially no change in muscle potassium as bath potassium was lowered from 5.5 to 1.1 mEq per L. By contrast, comparable changes in plasma potassium in intact potassium-deficient rats were found to be accompanied by large reductions in diaphragm potassium.

It is concluded that reduction in external potassium concentration alone is insufficient to produce the loss of muscle potassium observed in potassium-deficient rats. The striking difference between the *in vivo* and *in vitro* behavior of skeletal muscle cannot be explained by changes in extracellular acid-base balance; other as yet unidentified factors must play a role.

ACKNOWLEDGMENT

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THE EFFECT OF ETHANOL ON FATTY ACID METABOLISM; STIMULATION OF HEPATIC FATTY ACID SYNTHESIS *IN VITRO* *

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Fatty infiltration of the liver is a common finding in alcoholic patients, but the mechanisms responsible for the accumulation of hepatic fat are not clear (2). In the present investigation, an attempt was made to clarify this relationship by studying the effect of ethanol on fatty acid metabolism in rat liver *in vitro* (3).

MATERIALS AND METHODS

Seventy-one male Sprague-Dawley rats weighing from 250 to 400 g were maintained on Purina Laboratory Chow, except for a 24 hour period immediately preceding the experiment. During this period, they were given 36.5 g glucose per kg together with 7.5 g ethanol per kg or, in the experiment shown in Table III, 50 g per kg fructose, administered by means of 5 gastric tube feedings of equal volume. Approximately 3 hours after the last gastric feeding, the animals were decapitated. The liver was quickly removed, and slices were prepared in the cold with a Stadie-Riggs slicer (4). The C^{14}-labeled [1] and unlabeled substrates were dissolved in 5 ml isotonic Krebs-Ringer bicarbonate buffer, pH 7.4 (6), and the final concentration of the substrates was expressed as millimoles per liter. An aliquot of each incubation mixture was added to a toluene solution containing 12 per cent methanol, 0.4 per cent diphenyloxazole (DPO) and 0.005 per cent 1,4-di-[2-(5 phenyloxazolyl)] benzene (POPOP) (7) and the radioactivity counted in a Packard liquid scintillation counter. Randomized liver slices (approximately 0.5 g) were suspended in the incubation mixture in the main compartment of incubation flasks (8). The flasks were sealed with serum caps, gassed five minutes with a mixture of 95 per cent O_2 and 5 per cent CO_2, and then incubated for 3 hours at 37.5° C in a Dubnoff shaking water bath. Rat epididymal fat pads (0.5 to 1.0 g) were incubated in the same manner. At the end of the incubation, 0.2 ml of 10 N sulfuric acid was injected with a needle through the serum cap into the main compartment, and 1 ml of alkaline hyamine (9) into the center well. The flasks were then shaken for 30 minutes in an ice bath to trap the evolving CO_2 in the hyamine. The hyamine-$C^{14}O_2$ solution was transferred into a volumetric flask and made up to 5 ml with toluene; 2 ml of this solution was mixed with 16 ml of toluene containing 0.4 per cent DPO and 0.005 per cent POPOP (9), and the radioactivity was measured in a Packard liquid scintillation counter. To calculate the disintegrations per minute (dpm), the obtained counts were multiplied by the efficiency of the counter which was determined separately for each experiment with an internal C^{14} standard. $C^{14}O_2$ was expressed in dpm per gram of wet tissue or per milligram of tissue nitrogen.

The incubated tissue from each flask was homogenized in the incubation mixture, and an aliquot of this homogenate was used for determination of total nitrogen by the micro-Kjeldahl method (10). The rest of the homogenate was transferred to a screw-capped bottle, to which 0.4 ml of 90 per cent (wt/vol) potassium hydroxide per ml of homogenate was added. This mixture was saponified by autoclaving for 1 hour at 120° C. After cooling, ethanol was added to obtain a final concentration of 50 per cent. The mixture was vigorously shaken with 20 ml petroleum ether for 10 minutes to remove nonsaponifiable material. After repeating the extraction with petroleum ether 3 times, the ethanol remaining in the aqueous phase was removed by heating on a steam bath, and the pH was reduced to below 3.0 with concentrated sulfuric acid. After cooling, the fatty acids were extracted with 20 ml petroleum ether. The extraction was repeated 3 times, and the ether extracts were pooled and washed twice with water. An aliquot of the washed ether extract was evaporated under a nitrogen stream, and the dry residue was used for estimation of total fatty

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[1] The following C^{14}-labeled substrates were used: acetate-1-C^{14}, acetate-2-C^{14}, ethanol-1-C^{14}, ethanol-2-C^{14}, and palmitate-1-C^{14}, the latter being bound to crystalline bovine albumin (5). The various substrates were purchased from New England Nuclear Corp., Boston, Mass., and Tracerlab, Inc., Waltham, Mass.

acids (11). The results were expressed in milligrams of palmitic acid per milligram of tissue nitrogen. Using this method, about 95 per cent of palmitic acid added to homogenized liver slices was recovered. Another dried aliquot was dissolved in toluene containing 0.4 per cent of DPO and 0.005 per cent of POPOP, and the radioactivity of the fatty acids was determined in a Packard liquid scintillation counter. The labeling of fatty acids was expressed in dpm per milligram of tissue nitrogen. To permit comparison of the results obtained with different substrates and substrate concentrations, incorporation of C^{14} into fatty acids was also expressed as percentage of the $C^{14}O_2$ evolved; labeling expressed in this way will subsequently be called "relative incorporation." In all experiments, control incubations were carried out with acid-inactivated liver slices.

In each experiment, tissues obtained from the same animal were used for comparison of the effect of the various substrates. The mean of the individual differences was calculated and its degree of significance was tested by the *t* test (12).

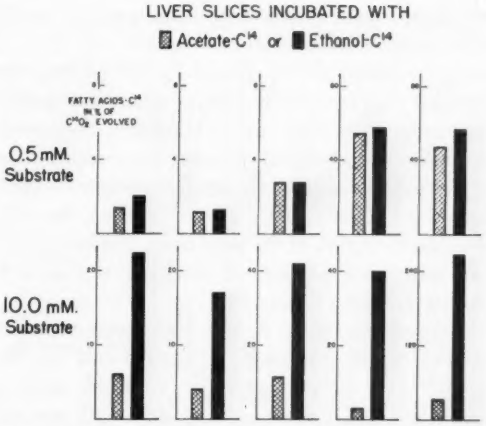
LIVER SLICES INCUBATED WITH
Acetate-C¹⁴ or Ethanol-C¹⁴
FATTY ACIDS-C¹⁴ IN % OF C¹⁴O₂ EVOLVED
0.5 mM. Substrate
10.0 mM. Substrate

FIG. 1. LABELING OF FATTY ACIDS IN LIVER SLICES INCUBATED WITH ACETATE-1,2-C^{14} OR ETHANOL-1,2-C^{14}. For both ethanol and acetate, the specific activity was 1.3 mc per mmole at the lower substrate concentration (0.5 mM), and 0.033 mc per mmole at the higher substrate concentration (10 mM). The results are expressed as fatty acids-C^{14} in per cent of $C^{14}O_2$ evolved (relative incorporation).

RESULTS

1. Total fatty acid content of liver slices incubated with ethanol, acetate or glucose. After incubation for 3 hours, the total fatty acid content of rat liver slices averaged 0.885 mg palmitic acid per mg tissue nitrogen when incubated with 10 mM ethanol, and 0.805 mg palmitic acid on incubation with 10 mM acetate. In 17 paired determinations, the mean difference was 0.080 ± 0.029 mg palmitic acid per mg tissue nitrogen ($p < 0.02$).

In nine instances, slices were incubated with either 10 mM ethanol or 5 mM glucose. At the end of the incubation period, the total fatty acid content of the slices was on the average 0.901 mg palmitic acid per mg of tissue nitrogen in the presence of ethanol, and 0.831 mg palmitic acid, with glucose. The mean difference was 0.070 ± 0.021 mg palmitic acid per mg of tissue nitrogen ($p < 0.02$).

2. Incubation of liver slices and adipose tissue with acetate-1,2-C^{14} or ethanol -1,2-C^{14}. As seen

TABLE I

Incubation of rat liver slices *

With 0.33 μc/ml acetate-1-C^{14} and 10 mM unlabeled acetate			With 0.33 μc/ml acetate-1-C^{14} and 10 mM unlabeled ethanol		
$C^{14}O_2$	Fatty acids-C^{14}		$C^{14}O_2$	Fatty acids-C^{14}	
dpm/mg N	*dpm/mg N*	*% of $C^{14}O_2$* †	*dpm/mg N*	*dpm/mg N*	*% of $C^{14}O_2$*
25,940	1,260	4.9	9,300	14,500	155.9
19,940	1,260	6.3	15,330	6,570	42.9
33,540	2,870	5.8	9,950	1,180	11.9
26,390	1,390	5.3	15,810	2,090	13.2
34,180	1,850	5.4	19,060	6,040	31.7
2,340	230	9.6	7,170	1,340	18.7
19,840	3,660	18.4	20,700	6,190	29.9
34,070	1,700	5.0	18,920	6,290	33.2
25,560	400	1.6	21,550	2,740	12.7
20,470	250	1.2	15,580	3,400	21.9
32,090	970	3.0	11,730	5,270	44.9
5,030	1,640	32.7	12,740	23,070	181.1

* In this and in all subsequent tables, results given on the same horizontal line were obtained with tissue from the same rat.

† C^{14} in fatty acids × 100/C^{14} in CO_2.

in Figure 1, at a substrate concentration of 0.5 mM, relative incorporation of acetate-1,2-C^{14} (1.3 mc per mmole) and of ethanol-1,2-C^{14} (1.3 mc per mmole) into fatty acids of liver slices was similar for each individual rat, although considerable variation was observed between different animals.

With a substrate concentration 20 times higher, however (10 mM; 0.033 mc per mmole), the relative incorporation of the label from ethanol-1,2-C^{14} into fatty acids was 3 to 12 times higher than with acetate-1,2-C^{14} (Figure 1).

Adipose tissue of 8 rats was incubated with either 0.5 mM ethanol-1,2-C^{14} or 0.5 mM acetate-1,2-C^{14} (1.3 mc per mmole). With ethanol-C^{14}, much less $C^{14}O_2$ was produced than with acetate-C^{14}, the respective values being 156 ± 58 dpm per mg of tissue nitrogen against 1,787 ± 306 dpm.

3. Effect of unlabeled substrates on the metabolism of acetate-1-C^{14} in liver slices and adipose tissue. The results in Tables I and II indicate that on incubation of liver slices with 0.33 μc per ml acetate-1-C^{14} (specific activity of the undiluted acetate-1-C^{14}: 1 mc per mmole), incorporation of the label into fatty acids was approximately four times higher in the presence of 10 mM unlabeled ethanol than with 10 mM unlabeled acetate ($p < 0.001$). With 10 mM unlabeled ethanol, the relative incorporation of the label into fatty acids was approximately six times greater than with 10 mM unlabeled acetate and three times greater than with 5 mM unlabeled glucose (Figure 2).

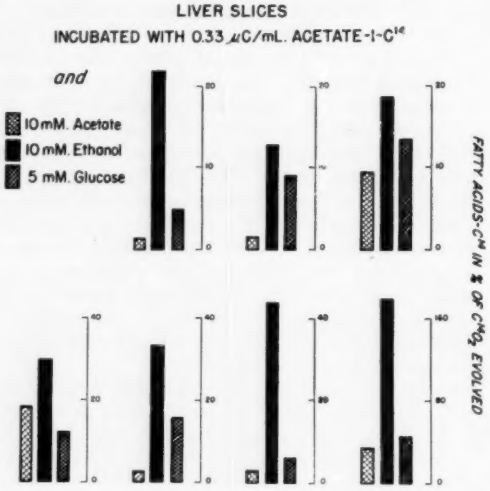

FIG. 2. EFFECT OF UNLABELED ACETATE, ETHANOL OR GLUCOSE ON THE RELATIVE INCORPORATION OF ACETATE-1-C^{14} INTO FATTY ACIDS IN LIVER SLICES.

As seen in Table II, incubation of liver slices in the presence of 0.6 mM methylene blue resulted in decreased incorporation of the label into fatty acids in all experiments. It reduced in part the stimulatory effect of ethanol on the relative incorporation of the label into fatty acids.

In adipose tissue, no stimulatory effect was observed with ethanol, while glucose enhanced acetate-1-C^{14} incorporation into fatty acids (Figure 3).

TABLE II

Effect of methylene blue on incubation of rat liver slices

Rat	With 0.33 μc/ml acetate-1-C^{14} and 10 mM unlabeled acetate			With 0.33 μc/ml acetate-1-C^{14} and 10 mM unlabeled ethanol		
	$C^{14}O_2$	Fatty acids-C^{14}		$C^{14}O_2$	Fatty acids-C^{14}	
	dpm/mg N	*dpm/mg N*	*% of $C^{14}O_2$**	*dpm/mg N*	*dpm/mg N*	*% of $C^{14}O_2$*
Without methylene blue						
a	35,410	5,840	16.5	22,620	25,520	112.8
b	47,920	9,040	18.9	30,190	20,120	66.6
c	38,540	4,804	12.5	28,990	9,910	46.1
d	44,820	1,640	3.7	34,520	13,540	39.2
e	15,540	317	2.0	10,570	9,100	86.1
With 0.6 mM methylene blue						
a	25,590	710	2.8	29,560	3,500	11.8
b	29,530	1,240	4.2	33,070	4,270	12.9
c	10,790	430	3.9	27,910	2,000	7.2
d	36,690	230	0.6	41,890	920	2.2
e	8,850	132	1.5	21,180	890	4.2

* C^{14} in fatty acids × 100/C^{14} in CO_2.

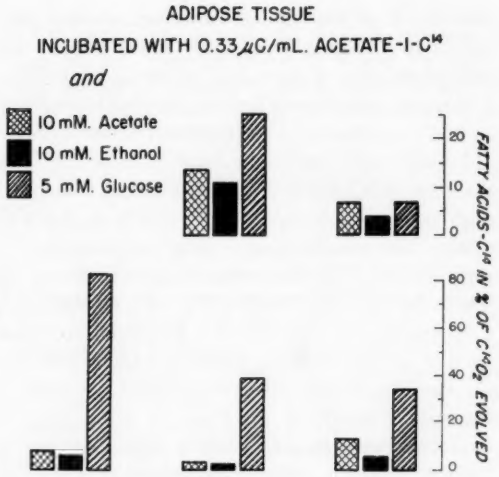

FIG. 3. EFFECT OF UNLABELED ACETATE, ETHANOL OR GLUCOSE ON THE RELATIVE INCORPORATION OF ACETATE-1-C^{14} INTO FATTY ACIDS IN ADIPOSE TISSUE.

The results of Table III indicate that in the presence of unlabeled sorbitol, incorporation of the label into fatty acids of liver slices was two to five times higher than with equimolar amounts of unlabeled fructose.

4. Effect of ethanol on the $C^{14}O_2$ production from palmitate-1-C^{14} and acetate-1-C^{14} in liver slices. Liver slices were incubated with 0.03, 0.04 or 0.06 μc per ml palmitate-1-C^{14} (1 mc per mmole), supplemented with either 10 mM unlabeled acetate or 10 mM unlabeled ethanol.

On incubation with ethanol, the average $C^{14}O_2$ formation in 14 experiments was 30,900 dpm per g of tissue and on incubation with acetate, 58,500 dpm. The mean difference was 27,600 ± 10,500 dpm per g of tissue ($p < 0.05$). When, under

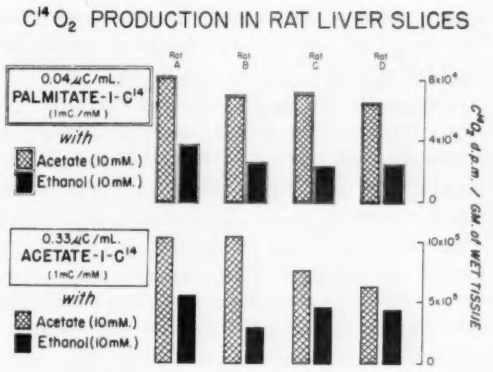

FIG. 4. EFFECT OF UNLABELED ETHANOL OR ACETATE ON $C^{14}O_2$ PRODUCTION BY RANDOMIZED LIVER SLICES, INCUBATED WITH EITHER ACETATE-1-C^{14} OR PALMITATE-1-C^{14}.

similar experimental conditions, palmitate-1-C^{14} was replaced by acetate -1-C^{14} (Tables I and II), the average $C^{14}O_2$ production was 304,700 dpm per mg of tissue nitrogen in the presence of unlabeled ethanol and 461,600 dpm with unlabeled acetate. The mean difference in 17 experiments was 156,900 ± 9,200 dpm per mg of tissue nitrogen ($p < 0.001$).

In four instances when randomized liver slices obtained from the *same* animal were used to study the effect of 10 mM ethanol on $C^{14}O_2$ production from both palmitate-1-C^{14} and from acetate-1-C^{14}, the reduction in $C^{14}O_2$ formation was comparable with both labeled substrates (Figure 4).

DISCUSSION

The total fatty acid content of liver slices incubated for 3 hours with ethanol was found to be significantly higher as compared to incubation

TABLE III

Incubation of rat liver slices

With 0.33 μc/ml acetate-1-C^{14} and 60 mM unlabeled fructose			With 0.33 μc/ml acetate-1-C^{14} and 60 mM unlabeled sorbitol		
$C^{14}O_2$	Fatty acids-C^{14}		$C^{14}O_2$	Fatty acids-C^{14}	
dpm/mg N	*dpm/mg N*	*% of $C^{14}O_2$**	*dpm/mg N*	*dpm/mg N*	*% of $C^{14}O_2$*
58,180	28,920	49.7	55,660	69,640	125.1
69,280	19,120	27.5	73,280	70,860	96.7
62,300	20,670	33.2	72,890	53,380	73.2
62,930	32,070	51.0	71,090	81,960	115.2
33,840	11,810	34.9	33,180	49,920	150.0
37,590	7,320	19.5	49,670	46,920	94.5

* C^{14} in fatty acids × 100/C^{14} in CO_2.

with glucose or acetate. In the subsequent experiments, an attempt was made to clarify the mechanism responsible for this finding.

The results in Figure 1 indicate that at a substrate concentration of 0.5 mM, the relative incorporation of ethanol-C^{14} and acetate-C^{14} into hepatic fatty acids was similar, while with a substrate concentration 20 times higher, relative incorporation into fatty acids was 3 to 12 times higher with ethanol-C^{14} as compared with acetate-C^{14}. Similarly, when liver slices were incubated with acetate-C^{14} and either 10 mM unlabeled ethanol or unlabeled acetate (Table I), ethanol enhanced the incorporation of acetate-C^{14} into fatty acids and reduced the production of $C^{14}O_2$.

Interpretation of these findings is complicated by the variable dilution of the label by the unlabeled substrates and also by possible differences in the rate of metabolism between ethanol and acetate. However, since both for incorporation into fatty acids and for conversion to CO_2 via the tricarboxylic acid cycle (TCA), acetate and ethanol pass through a common acetyl-CoA pool (13) and since the total disposition of acetate-C^{14} into both lipids and CO_2 was approximately of the same order of magnitude (Table I), the present findings may suggest that on incubation with ethanol, utilization of acetyl-CoA is shifted in such a way that a relatively larger fraction is incorporated into fatty acids while less is metabolized via the TCA cycle.

In adipose tissue, where lack of alcohol dehydrogenase activity prevents significant oxidation of ethanol (3, 14), ethanol failed to stimulate incorporation of acetate-C^{14} into fatty acids (Figure 3).

In the liver, ethanol is oxidized by alcohol dehydrogenase (15–17) with concomitant reduction of diphosphopyridine nucleotide (DPN) to DPNH (18). *In vivo* and *in vitro*, ethanol was found to reduce the hepatic DPN/DPNH ratio (19, 20).

Decreased concentration of DPNH in the liver was shown to be associated with reduced fatty acid synthesis (21), while in cell-free liver extracts, addition of exogenous DPNH was found to stimulate fatty acid synthesis (22, 23). These findings suggest that ethanol may enhance incorporation of acetate-C^{14} into hepatic fatty acids through the excess DPNH formed on ethanol oxidation. It is not known, however, whether this occurs by reducing TCA cycle activity or by direct stimulation of fatty acid synthesis, or both.

Supportive evidence for this concept is obtained by the experiments with sorbitol-fructose (Table III) and with methylene blue (Table II). In the liver, oxidation of sorbitol to fructose is coupled with concomitant reduction of DPN to DPNH (24). The increased incorporation of acetate-C^{14} into fatty acids in the presence of sorbitol may be due to the shift in DPN/DPNH ratio (Table III). Conversely, the presence of a hydrogen acceptor, such as methylene blue, would be expected to offset the stimulatory effect of ethanol, such as is shown in Table II.

It has been shown *in vitro* that addition of DPNH results in reduced oxidation of TCA cycle intermediates (25). Thus, the excess DPNH generated on ethanol oxidation may account for the reduced formation of $C^{14}O_2$ from acetate-C^{14} (Tables I and II) and from palmitate-1-C^{14} (Figure 4). The possibility cannot be excluded with certainty, however, that in addition to stimulating fatty acid synthesis, ethanol may exert an inhibitory effect on fatty acid oxidation.

CONCLUSIONS AND SUMMARY

1. The total fatty acid content of liver slices is significantly higher when incubated with ethanol than with glucose or acetate.

2. The incorporation of acetate-C^{14} into fatty acids of liver slices is stimulated by ethanol, as compared with acetate or glucose.

3. In adipose tissue, where only small amounts of ethanol are oxidized, no stimulation of fatty acid synthesis by ethanol is observed.

4. In liver slices incubated with ethanol, $C^{14}O_2$ production from acetate-1-C^{14} and from palmitate-1-C^{14} is depressed to a similar extent.

5. Oxidation of ethanol in the liver results in reduction of DPN and a shift in incorporation of acetate-C^{14} toward fatty acid synthesis. Another DPNH generating system (sorbitol-fructose) reproduces this effect, while methylene blue reverses it. These findings suggest that the stimulation of hepatic fatty acid synthesis by ethanol may be the result of the excess DPNH formed on ethanol oxidation.

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STUDIES ON PYROGENIC STEROIDS. I. SEPARATION, IDENTIFICATION, AND MEASUREMENT OF UNCONJUGATED DEHYDROEPIANDROSTERONE, ETIOCHOLANOLONE, AND ANDROSTERONE IN HUMAN PLASMA *

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Previous studies have shown that endotoxin (1–4), viruses (5–7), and pyrogenic substances from polymorphonuclear leukocytes (8–11) are capable of producing fever. A different group of pyrogenic substances was described by Kappas, Soybel, Fukushima and Gallagher (12), who reported that certain C_{19} and C_{21} steroids with a 3α-hydroxy, 5β configuration produce fever. The prototype compound is etiocholanolone [1] (13–16). Four to 8 hours after intramuscular or intravenous administration of this compound to volunteers, the temperature began to rise, reached a peak (103° to 105° F) at 12 hours, and generally subsided within 24 hours. Chills, anorexia, headache, malaise, myalgia, arthralgia, and leukocytosis accompanied the reaction.

Recently, Bondy, Cohn, Herrmann and Crispell (16) demonstrated unconjugated [2] etiocholanolone in the plasma of two patients during febrile attacks of etiocholanolone fever.[3] When the patients were afebrile and asymptomatic, unconjugated plasma etiocholanolone was not detected. The 24-hour urinary 17-ketosteroid patterns were normal when the patients were without symptoms. However, etiocholanolone was the only major 11-deoxy-17-ketosteroid isolated during the symptomatic interval.

Since the plasma clearance of 11-deoxy-17-ketosteroids, such as androsterone and etiocholanolone (18) is rapid (20 minute half-life), previous attempts to recover these steroids and dehydroepiandrosterone in an unconjugated form from plasma of normal subjects has been discouraging, since at most, only trace amounts were present (19, 20).

However, Savard (21) was able to isolate and identify (but not measure) unconjugated androsterone from 100 L of extracorporeal dialysis fluid obtained from a 22 year old patient with acute tubular necrosis and anuria. Moreover, Tamm, Beckmann and Voigt (22) analyzed 200 to 300 ml of normal human sera and found 17 to 61 μg per 100 ml of total unconjugated 17-ketosteroids, measured by micro-Zimmermann reaction, after chloroform extraction, NaOH wash, and Girard separation. However, no separation of 11-oxy- and 11-deoxy-17-ketosteroid was attempted. On the other hand, Migeon (23) extracted 500 ml of normal human plasma and reported that "there was probably not more than 1 μg per 100 ml each of unconjugated androsterone and dehydroepiandrosterone."

In the present study unconjugated plasma 17-ketosteroids were measured in normal subjects and in patients with fever of known origin, familial Mediterranean fever and etiocholanolone fever. Unconjugated plasma androsterone, dehydroepiandrosterone, and etiocholanolone were separated, measured and identified as 2,4-dinitrophenylhydrazone derivatives. An isotope dilution method

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[1] The following trivial names and abbreviations are used: etiocholanolone, ETIO (3α-hydroxyetiocholane-17-one); androsterone, ANDRO (3α-hydroxyandrostane-17-one); dehydroepiandrosterone, DHEA (3β-hydroxy-5-androstene-17-one); etiocholanolone fever, EF; familial Mediterranean fever, FMF.

[2] The term *unconjugated* refers to those steroids extracted with an organic solvent (viz., chloroform) without previous hydrolytic procedures.

[3] It was suggested that this type of fever be called etiocholanolone fever in order to distinguish this syndrome from familial Mediterranean fever on the one hand and the undifferentiated periodic fevers on the other (17).

with tritium-labeled etiocholanolone, androsterone, and dehydroepiandrosterone was used to correct for losses during the procedures. The method is sufficiently sensitive to be useful in measuring unconjugated neutral 17-ketosteroids in plasma.

MATERIALS AND METHODS

Subjects

1. Afebrile subjects. Five hundred ml of heparinized blood was collected between 8 and 10 a.m. from 4 healthy men and 2 healthy women, ranging in age from 23 to 45 years. The plasma was separated by centrifugation within 15 minutes of collection and frozen until analyzed. Ten determinations were performed on 110- to 120-ml portions of this plasma. In addition, 3,000 ml of fresh plasma, obtained from untreated patients with polycythemia vera, was used for isotopic and chemical recovery experiments.

2. Febrile patients. Unconjugated neutral 17-ketosteroids were measured on 12 hospital inpatients with fever (101° F or higher) of known origin. The clinical data are listed in Table I. Heparinized blood was obtained at the time of the indicated temperature elevation. The plasma was frozen until processed. Liver and kidney function tests and an endocrinological evaluation revealed no abnormalities. M.J. (ulcerative colitis, Table I) had received no steroid therapy for at least 3 months prior to entry to the hospital. None of the other patients had ever received steroids.

3. Patients with etiocholanolone fever and familial Mediterranean fever. The clinical data are listed in Table II. The patients with familial Mediterranean fever presented the classical family history, signs and symptoms described by Heller, Sohar and Sherf (24) and Lawrence and Mellinkoff (25). The patients with etiocholanolone fever were not of Mediterranean or Middle Eastern origin. Repeated cultures, skin tests, and precipitin and agglutination tests for a wide variety of infectious agents were unrevealing. Liver function tests, lymph node and liver biopsies were within normal limits. Total and differential white blood counts were normal except during acute febrile attacks, when a polymorphonuclear leukocytosis with a shift to the left (without eosinophilia) usually occurred.

Plasma samples were collected during the prodrome of an attack, while the temperature was rising (100° to 102° F). Plasma was also collected during an afebrile and asymptomatic interval when the patients were not receiving therapy. These plasma samples were also frozen until analyzed.

Preparation of radioactive 17-ketosteroids for isotope dilution

Two mg of etiocholanolone, mp 149° to 151° C, and 2.5 mg of androsterone,[4] mp 181° to 183° C, were each exposed to 3 curies of tritium at 27° C and 0.39 atm for 14 days.[5]

The readily labile tritium was removed by a modification of a method described by Peterson (27) using 250 to 500 ml of methanol. After the methanol was removed by vacuum distillation, the dry impure steroid was dissolved in 20 ml of freshly distilled chloroform. One ml of 0.05 N NaOH was added, mixed, and the alkaline chloroform extract washed three times with 1 to 2 ml of distilled water. The aqueous phase was carefully removed by aspiration and the chloroform extract evaporated to dryness under nitrogen. The residue was dissolved in 1 ml of ethyl acetate: methanol (2:1) and applied as a streak adjacent to reference compounds of androsterone and etiocholanolone on washed (28) Whatman no. 1 chromatographic papers in the heptane: propy-

[4] Etiocholanolone and androsterone were generously donated by Dr. Seymour Lieberman, Dept. of Biochemistry, Columbia University, New York, N. Y.

[5] The Wilzbach procedure (26) was performed by the New England Nuclear Corp., Boston, Mass.

TABLE I

Clinical data in patients with fever of known origin

Patient	Sex	Age	Diagnosis	Rectal temp.	Vol. of plasma
		yrs		*° F**	*ml*
M.S.	F	22	Viral pneumonia	105	28
B.B.	F	28	Acute follicular tonsillitis	106	63
L.C.	M	29	Acute cholecystitis	103	41
H.J.	F	33	Viral pneumonia	102.5	40
M.R.	M	36	Acute gasteroenteritis	104	55
E.N.	F	44	Malignant melanoma	101.2	45
M.J.	F	45	Ulcerative colitis	101	65
E.W.	M	51	Bronchopneumonia	103	45
S.G.	F	61	Lung abscess	102	50
A.C.	M	64	Acute appendicitis	103	55
M.I.	M	68	Chronic bronchitis; emphysema	101	47
M.Y.	F	73	Bronchopneumonia	103	33

* Plasma samples were obtained at the time of the indicated temperature. No sample contained as much as 0.5 μg etiocholanolone per 100 ml of plasma.

TABLE II

Clinical data and unconjugated plasma 17-ketosteroid levels in patients with etiocholanolone fever and familial Mediterranean fever *

Patient	Sex	Age	Diag.	Age at onset	Freq. of attacks	Duration, attacks	Temp.†	Vol. of plasma	Radioactive recovery			Corrected plasma values		
									DHEA	ETIO	ANDRO	DHEA	ETIO	ANDRO
		yrs		*yrs*	*wks*	*days*	° F	*ml*	%			*μg/100 ml*		
T.N.	M	10	EF	8	4–5	3–5	98.7	52	57.8	67.2	80.0	<0.5	1.0	2.0
							102.0	53	56.8	52.6	73.3	7.3	6.5	0.8
W.H.	M	28	EF	13	3–4	3–4	98.6	67	51.8	62.8	34.0	0.6	0.5	2.6
							103.0	62	65.0	60.2	54.1	4.1	2.0	5.6
H.P.	M	30	EF	10	4–6	3–4	98.6	50	77.1	59.7	55.4	0.5	<0.5	0.8
							101.8	30	26.1	87.6	61.0	28.3	6.7	1.6
M.K.‡	M	44	EF	18	3–4	3–5	98.5	44	40.0	55.7	22.1	0.6	<0.5	1.0
							102.0	23	59.8	70.6	83.0	7.2	7.3	1.1
V.E.	M	48	EF	34	4	3–4	98.8	50	75.1	81.6	47.3	.5	0.5	0.6
							102.5	50	61.4	77.9	62.2	10.4	4.1	8.7
W.S.	M	28	FMF	23	irreg.	2	98.6	50	54.7	61.5	61.8	1.5	<0.5	2.3
							103.0	50	48.0	61.4	50.9	13.3	<0.5	1.5
A.N.	M	32	FMF	17	4–6	4–5	*103.0*	50	51.2	48.2	§	0.8	0.8	§
R.Q.	F	42	FMF	27	irreg.	2–3	*102.0*	100	76.1	70.7	§	0.7	0.7	§

* For abbreviations in this and following tables, see footnote[1] to text.
† Plasma samples were collected during attacks at the time of the italicized temperature measurements.
‡ This patient was reported (16) and subsequently reinvestigated.
§ These samples were lost.

lene glycol system (29).[6] The mobility of the radioactive peak, located by isotopic scanning, coincided with the mobility of the Zimmermann positive staining reference standards. Most of the radioactive impurities were more polar and easily separated from the tritium-labeled androsterone and etiocholanolone. The radioactive steroid area corresponding to the reference standard was eluted four times with 10-ml washes of the ethyl acetate: methanol (2:1) mixture, dried under nitrogen, and rechromatographed along with appropriate standards in the 1:1 heptane:96 per cent methanol system (30). The radioactive steroids were located and eluted as described. The isolated tritium-labeled steroids were purified by two additional paper chromatographic procedures in the same systems.

Dehydroepiandrosterone-7α-H^3-acetate [7] was converted to the free steroid by hydrolysis with acetyl cholinesterase (Nutritional Biochemicals Corp.) by the method of Peterson and colleagues (31). The free steroid was washed, dried, extracted, and purified by paper chromatography as described.

Constant specific activity was observed following reverse isotope dilution of the free tritium-labeled steroids using paper chromatography, and column chromatography of the 2,4-dinitrophenylhydrazone derivatives (32) on 3.9 and 5.0 per cent alumina micro-columns as described below (Table III). The constant specific activities were interpreted as evidence for the radiochemical purity of the steroids.

Plasma 17-ketosteroids

Separation. Approximately 5,000 cpm (0.2 ml) of tritium-labeled etiocholanolone (5.2 μc per μg), androsterone (2.6 μc per μg), and dehydroepiandrosterone (4.4 μc per μg) were pipetted into a 250 ml centrifuge tube and evaporated to dryness. From 45 to 50 ml of plasma was added and the tube was shaken for about 2 minutes to insure complete mixing of the tritium-labeled steroids and the plasma. Two ml of 1 N NaOH per 40 ml of plasma was added, mixed, and the plasma extracted three times with 2 vol of freshly distilled chloroform. Emulsions were broken by centrifugation. The chloroform extract was dried *in vacuo* and transferred quantitatively with chloroform to washed Whatman no. 1 filter paper in the heptane:96 per cent methanol system of Bush and Willoughby (30). Twenty-five μg each of reference compounds androsterone, etiocholanolone, and dehydroepiandrosterone were applied together in a single spot, on each side of the extract area. A sheet of filter paper 19 cm wide conveniently accommodated three reference area spots and two extract spots. The paper was equilibrated for 4 hours and the mobile phase allowed to de-

[6] Paper chromatography is carried out in a room with constant temperature of 20° C and relative humidity of less than 50 per cent.

[7] Dehydroepiandrosterone-7α-H^3-acetate, SA 2.46 mc per mg, lot no. 15-258-9, from New England Nuclear Corp.

TABLE III

Specific activities of standard tritium-labeled 17-ketosteroids following reverse isotope dilution

Method	Solvent system	Specific activity		
		DHEA	ETIO	ANDRO
		cpm/μg		
Paper chromatography	Free compounds*			
	Heptane: 96% methanol (1:1)	57,781	74,475	78,024
Column chromatography	2,4-Dinitrophenylhydrazone derivatives			
3.9% Alumina	Benzene: chloroform (4:1)	58,685	77,369	82,450
5.0% Alumina	Benzene: chloroform (4:1)	59,700	77,694	83,390

* The radioactive steroids were separated by paper chromatography after the addition of 10 μg of each reference standard. The tritium-labeled free steroids were converted to the 2,4-dinitrophenylhydrazones and measured spectrophotometrically. Radioactivity was determined with corrections for quenching. The (formed)steroid 2,4-dinitrophenylhydrazones were rechromatographed on columns and specific activity determined as described.

velop for 12 to 14 hours, after which the paper was air-dried. The three standard strips were cut off and the reference compounds located by staining with Zimmermann reagent, according to the technique of Migeon and Plager (20). Appropriate areas corresponding to the reference standards and paper blanks were cut out, placed in glass-stoppered test tubes, and eluted with 4 ml of absolute ethanol for 45 minutes at 37° C. Paper blanks were usually obtained below the androsterone (least polar compound) area. However, if the androsterone migrated to the bottom of the paper, a paper blank was taken either above the dehydroepiandrosterone (most polar compound) area, well away from any ultraviolet-absorbing material or below the etiocholanolone area.

Measurement. Two-ml portions of the eluates were pipetted into acid-cleaned 15 × 85 mm Pyrex tubes and dried *in vacuo* for the 2,4-dinitrophenylhydrazine reaction (32). The samples were dissolved in 0.5 ml of absolute ethanol and 0.04 ml of freshly prepared 2,4-dinitrophenylhydrazine reagent (2.0 mg per ml of absolute ethanol acidified with 0.05 ml 12 N HCl) was added. The mixture was allowed to stand overnight at room temperature. The following day, 0.5 ml distilled water and 0.5 ml Benedict's reagent were added. The tubes were heated for 15 minutes in a vigorously boiling water bath, and, after cooling, 0.5 ml of freshly distilled chloroform was added. The tubes were stoppered quickly to prevent evaporation of the chloroform. Transfer of the steroid-dinitrophenylhydrazone to the chloroform layer was facilitated by vibrating the test tube containing the Benedict reagent: chloroform mixture for 1 minute with a Dremel hand drill, model 2 (Dremel Mfg. Co. Racine, Wis.), which was fitted with a one-eighth inch rod-like bit, bent at a 20° to 25° angle. The tubes were allowed to stand at room temperature for 1 hour. The aqueous layer was carefully removed by aspiration. The chloroform layer (0.5 ml) was transferred to 0.8 ml microcuvets with 1.0 cm light path, and absorbance was measured at 368 mμ in a Beckman model DU spectrophotometer. A standard curve was constructed on each occasion with etiocholanolone-2,4-dinitrophenylhydrazone. Since androsterone and dehydroepiandrosterone-2,4-dinitrophenylhydrazones have the same absorbance maxima and extinction coefficients at 368 mμ (32), these compounds may be substituted in constructing the standard curve. The absorbance followed Beers' law from 0.3 μg (A = 0.030) to 3.0 μg (A = 0.300). The optical density of the samples was at least 2.5 to 4 times that of the paper blanks.[8]

A 0.5 ml portion of the ethanol eluate was counted to a standard error of ± 2 per cent in a liquid phosphor scintillation counter (Technical Measurements Co., model LP-2, New Haven, Conn.) which had a counting efficiency of 13 to 15 per cent for tritium. Since aliquots of the eluates from the paper blanks had only background radioactivity, no radioactive correction for paper blanks was necessary. The final value was calculated, taking into account the absorbance of the blank, the dilution factor, and the isotope recovery (28).

RESULTS

Plasma determinations. Normal values and isotopic and chemical recoveries are listed in Table IV. Twelve patients with fever of known cause (Table I) had less than 0.5 μg per 100 ml unconjugated etiocholanolone, dehydroepiandrosterone and androsterone.

[8] It has been observed that the steroid 2,4-dinitrophenylhydrazones can be further purified by the method of microalumina chromatography. In this way, the mean ± standard deviation elution blank readings at 368 mμ are A = 0.005 ± 0.002. After chemical measurements are performed, radioactive recovery is determined by direct plating at infinite thinness of the steroid-2,4-dinitrophenylhydrazones on planchets and counting the tritium in a windowless gas-flow counter (Automatic Sample Changer, model C 100 B, Nuclear-Chicago Corp., Des Plaines, Ill.). The efficiency for tritium is 28 to 30 per cent. This modification has increased the sensitivity from 0.5 μg to 0.2 μg per 100 ml.

TABLE IV

Normal unconjugated plasma 17-ketosteroid values; isotopic and chemical recoveries

	Range [10]*	Isotopic recovery [92] Mean ±SD	Chemical recovery [12]† Mean ±SD
	μg/100 ml	%	%
DHEA	<0.5‡ − 1.2	70 ± 10.4	81.0 ± 21.8
ETIO	<0.5 − 1.2	78 ± 13.0	66.7 ± 19.6
ANDRO	<0.5 − 0.8	67.6 ± 18.6	57.5 ± 21.8

* Figures in brackets represent the number of determinations performed. The normal range represents 10 measurements performed on the plasma of 6 volunteers.

† Twelve experiments were performed for each of the steroids, with the following amounts of reference compounds added to 100 to 200 ml of plasma: 1 μg [5]; 2 μg [3]; 5 μg [2]; 10 μg [1]; 20 μg [1]. The accuracy of the method is reproducible at the 1.0 μg per 100 ml level (mean ±SD = 1.0 ±0.25).

‡ The sensitivity of the method permits 0.5 μg to be interpolated from the standard curve. However, absorbance of the samples corrected for paper blank absorbance corresponding to lower than 0.5 μg cannot be interpolated. Thus the actual lower limit of the normal range has not been defined.

In Table II are listed the unconjugated plasma 17-ketosteroid measurements in three patients with familial Mediterranean fever and in five patients with etiocholanolone fever. It was not possible to perform duplicate determinations because of the limited amount of plasma available. As can be seen, etiocholanolone was not detected in the plasma of the three febrile patients with familial Mediterranean fever or in the plasma of the patients with etiocholanolone fever when they were afebrile.

However, elevated amounts of plasma etiocholanolone were detected during the febrile episodes of etiocholanolone fever. Moreover, a significant increase of plasma dehydroepiandrosterone was also demonstrated during the same symptomatic attack. During symptomatic episodes, three of the patients (W.H., H.P. and V.E.) had a 2-, 3- and 13-fold increase, respectively, of unconjugated androsterone, compared with the afebrile period.

Identification of plasma 17-ketosteroids. The 2,4-dinitrophenylhydrazone derivatives of unconjugated etiocholanolone, androsterone, and dehydroepiandrosterone isolated from all of the samples of plasma analyzed (a total of 10 L) were pooled separately and chromatographed on 1,000 mg of neutral aluminum oxide (Woelm) columns (12.5 × 0.3 cm). A stationary phase of benzene: petroleum ether (1:1) was used for the adsorption chromatography on 3.9 and 5.0 per cent hydrated alumina columns. The individual compounds were isolated by elution with benzene: chloroform, 4:1 (32). They were then reapplied to fresh 3.9 and 5.0 per cent alumina and eluted with 0.05 per cent ethanol in benzene (33). Identification of the steroid-dinitrophenylhydrazones was verified by characteristic position of the peaks on elution from the columns and maintenance of constant specific activities calculated on the basis of the absorbance of the eluted material at 368 mμ and the radioactivity, counted as described above, and corrected for quenching (Table V).

DISCUSSION

It is well established that isotopic labeling can be used to measure and to correct for losses of small amounts of steroids in analyzing plasma. The use of radioactive tracer(s) for correction is justified provided that certain conditions are met. First, the radioactive steroids must be as free as possible from contamination. This is especially important when the steroids are made radioactive by exposure to tritium gas. Second, radiochemical purity must be established and maintained. Third, the manipulations of extraction, paper chromatography, elution, and measurement of plasma steroids must affect equally both endogenous and added steroids. These conditions were strictly adhered to in the course of the development of the method described, and they facilitated an accurate chemical measurement.

Nevertheless, the sensitivity of the microchemical method prevented measurement below 0.5 μg per 100 ml of plasma since, if less than this amount of plasma 17-ketosteroid were present, it would produce an optical density reading indistinguish-

TABLE V

Specific activities of pooled unconjugated 17-ketosteroid 2,4-dinitrophenylhydrazones

Alumina % hydration*	Solvent system	Specific activity† DHEA	ETIO	ANDRO
		cpm/μg		
3.9	Benzene:$CHCl_3$ (4:1)	4,820	5,244	7,530
5.0	Benzene:$CHCl_3$ (4:1)	4,792	5,120	7,400
3.9	0.05 % Ethanol in benzene	4,780	5,100	7,320
5.0	0.05 % Ethanol in benzene	4,768	5,086	7,220

* The per cent hydrated alumina and solvent systems were employed in the order listed.

† Before column chromatography the specific activities of the pooled (10 L of analyzed plasma) tritium-labeled 17-ketosteroid 2,4-dinitrophenylhydrazones were: DHEA, 4,760; ETIO, 5,208; ANDRO, 7,480. The 2,4-dinitrophenylhydrazones were measured at 368 mμ in a spectrophotometer as previously described. Corrections for quenching were performed.

able from that of the paper blanks. Since the actual lower limit of the normal range has not been defined, statistical analysis of the data was not undertaken. However, the method did afford, by its sensitivity and reproducibility, an opportunity to investigate possible aberrations of androgen metabolism during fever.

Recently it was shown that the administration of certain steroids produces fever in human subjects. Kappas and co-workers (34) reported that the febrile response to steroids with a 3α-hydroxyl, 5β configuration differed from that produced by bacterial pyrogens (35). Steroid pyrogens, unlike bacterial pyrogens, were associated with a much longer latent period (4 to 6 hours) and did not produce tolerance after repeated administration (13). Moreover, these steroids did not produce fever in certain experimental animals (e.g., the dog or rabbit) which commonly respond to bacterial pyrogens (1, 2, 36). According to recent observations of Kappas and associates (34), the pyrogenic response to steroids was not due to the release of endogenous pyrogen.

This report describes five patients with acute attacks of etiocholanolone fever who had elevated unconjugated plasma etiocholanolone levels during the prodrome of chills, anorexia, abdominal and/or chest pain, myalgia, arthralgia, headache, and fever. Normal amounts of unconjugated etiocholanolone were detected in the plasma during the afebrile intervals. These observations support the thesis that the presence of the pyrogenic steroid may in some way be responsible for the development of this type of fever. However, the actual mechanism is still obscure. Unconjugated etiocholanolone was not found in the plasma of patients with fever of known origin or in three patients with familial Mediterranean fever. Therefore it seems unlikely that fever itself causes the presence of the steroid.

The plasma concentrations of dehydroepiandrosterone, an adrenal androgen (37), and (in three instances) androsterone were also increased in the febrile patients with etiocholanolone fever.

Although liver enlargement was present in the patients with etiocholanolone fever, liver function tests and biopsies were normal. Nevertheless, our data suggest that one hepatic defect occurs in this syndrome, i.e., a change in the pathway of androgen metabolism toward excessive etiocholanolone production. Tomkins (38) described two separate and specific steroid reductases which catalyze the reduction of the double bond on the A ring of steroids, to produce, in one case, 5β-steroids and, in the other, 5α-steroids such as androsterone. The acute attacks of etiocholanolone fever may be associated in some way with a decrease of the activity of the 5α-steroid reductases and concomitant increase of 5β-reductases. The analogous situation of increased etiocholanolone excretion, as opposed to androsterone excretion, has been described by Bradlow, Hellman, Zumoff and Gallagher (39) and Hellman and associates (40) in hypothyroidism. The administration of triiodothyronine reversed the clinical status and restored the androsterone, etiocholanolone (5α/5β) ratio to normal. Thyroid function measured by protein-bound iodine or butanol-extractable iodine and radioactive iodine uptake was within the normal range in the five patients with etiocholanolone fever. It would thus seem in the present study that this defect in C_{19} metabolism is unrelated to thyroid function. The precipitating causes and pathogenesis of etiocholanolone fever remain to be elucidated.

It has also been suggested (17) that an intermittent defect in conjugation might occur concomitantly with increased etiocholanolone production at the time of an attack of etiocholanolone fever. Kappas and colleagues (12) reported that substitution of an acetate radical on the third carbon atom of etiocholanolone inhibits pyrogenic activity. Kappas' observations suggest that etiocholanolone in plasma must be unconjugated if it is to account for the pyrogenic activity during attacks of etiocholanolone fever. Since the data indicate that unconjugated plasma etiocholanolone is associated with attacks, a possible aberration in conjugation has been hypothesized. In fact, preliminary data suggest that an attack is associated with decreased glucuronide conjugation of plasma tetrahydrocortisone and tetrahydrocortisol without increased β-glucuronidase activity.

Although the present studies have failed to find an increase in the unconjugated plasma etiocholanolone in patients with fever of known cause, only 12 such patients were studied. It is still too early to be certain that abnormalities of steroid metabolism do not occur in association with types of fever other than those studied in this brief series.

SUMMARY

A method is presented for the separation of unconjugated plasma 17-ketosteroids and micromeasurement of their 2,4-dinitrophenylhydrazone derivatives. An isotopic radiochemical dilution technique with pure tritium-labeled etiocholanolone, dehydroepiandrosterone, and androsterone is applied to correct for losses during the procedure. The normal plasma ranges are: etiocholanolone < 0.5 to 1.2 μg per 100 ml; androsterone, < 0.5 to 0.8 μg per 100 ml; dehydroepiandrosterone, < 0.5 to 1.2 μg per 100 ml. The total mean $\pm$ SD isotopic and chemical recoveries are 72 ± 14 and 67 ± 21 per cent, respectively.

Each of the measured 2,4-dinitrophenylhydrazone derivatives of unconjugated etiocholanolone, dehydroepiandrosterone, and androsterone was identified by characteristic elution mobility and maintenance of constant specific activity after four separate column chromatographic procedures.

Elevation of unconjugated plasma etiocholanolone (a known pyrogenic steroid) and dehydroepiandrosterone was demonstrated in five patients during attacks of etiocholanolone fever. In addition, three of the five patients had elevated unconjugated plasma androsterone levels. Dehydroepiandrosterone and etiocholanolone were within the normal range during the afebrile intervals. Unconjugated etiocholanolone was not detectable (< 0.5 μg per 100 ml) in the plasma of 12 patients with fever of known cause and 3 febrile patients with familial Mediterranean fever.

It has been suggested that in acute attacks of etiocholanolone fever, an intermittent decrease of hepatic 5α-reductases may be associated with a simultaneous increase in 5β-reductases, resulting in the production of excessive amounts of etiocholanolone at the expense of androsterone. Conclusive evidence of a defect in conjugation of 17-ketosteroids in etiocholanolone fever remains to be determined.

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BODY FLUID AND ELECTROLYTE COMPOSITION IN ARTERIAL HYPERTENSION. I. STUDIES IN ESSENTIAL, RENAL AND MALIGNANT HYPERTENSION *

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In previous studies in this laboratory it was found that the capacity to excrete sodium as indicated by the rate of excretion of an intravenously administered sodium load was significantly increased in essential and renal hypertension (1, 2). Sodium excretory capacity was reduced by antihypertensive therapy and was correlated significantly with the level of arterial pressure. However, the absence of a high degree of correlation, together with the ability of dietary sodium intake to influence the renal capacity to excrete sodium, suggested that in addition to an elevated blood pressure other factors such as an increase in body sodium and fluid volume might enhance sodium excretion in hypertensive individuals.

Studies in Cushing's syndrome and Addison's disease by other workers suggest that the level of adrenal cortical activity may influence not only the renal capacity to excrete sodium but also the arterial pressure as well as the body's content of fluids and electrolytes (3, 4). In line with these observations are recent reports indicating that an increase in aldosterone activity occurs in certain groups of hypertensive subjects (5–7).

To clarify the relationship of arterial hypertension to alterations in adrenal cortical function and in electrolyte and water metabolism, it was decided to study body fluid and electrolyte composition in various forms of hypertension, including that associated with primary aldosteronism (8). The present report describes the findings in essential, renal and malignant hypertension. The observations in primary aldosteronism are presented in a separate report (9).

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CLINICAL MATERIAL AND METHODS

Radioisotope dilution studies were performed in untreated ambulatory hypertensive patients and normal subjects who were paired as to age, sex, height, weight and body build. The dietary intake of salt as indicated by history and urinary sodium excretion over a 24 hour period was "unrestricted" and comparable in the hypertensive and the control subjects.

The 20 subjects with essential hypertension had sustained elevations of blood pressure with grade I to III retinopathy (Keith-Wagener) but without complicating nitrogen retention or congestive heart failure. However, 8 of these subjects were found to have enlarged left ventricles on roentgenographic examination.

The 10 subjects designated as having "renal hypertension" had a history of kidney disease which antedated the onset of the hypertension. Included in the group were 5 subjects with bilateral chronic pyelonephritis, 2 with polycystic kidney disease and 3 with glomerulonephritis. Their fundi showed grade I to III retinopathy whereas their blood urea nitrogen levels were normal except in 2 subjects. The left ventricle was enlarged in 5 subjects but no patient had complicating congestive heart failure.

The 7 hypertensive subjects with complicating heart failure had enlargement of the left ventricle with symptoms and signs of pulmonary congestion but without clinically detectable peripheral edema. The decholin circulation time was prolonged in all subjects but only 1 had a definite elevation of venous pressure. Two of the subjects had nitrogen retention and all showed grade II to III hypertensive retinopathy.

All 10 subjects who had malignant hypertension had grade IV retinopathy (papilledema) as well as marked elevation of blood pressure. In the group there were 4 subjects with chronic bilateral pyelonephritis, 3 with essential hypertension, 2 with glomerulonephritis, and 1 with renal arterial occlusion. Most of the subjects complained of fatigability and exertional dyspnea. They also had nitrogen retention as well as an enlarged left ven-

TABLE I

Essential hypertension *

Subject	Sex	Age	Body wt	BSA	BP	Fundi	BUN	Serum osm.	ECF ($S^{35}O_4$ space)	Na_s	K_s	Na_e/ body wt	K_e/ body wt
		yrs	*kg*	*m²*	*mm Hg*	*grade*	*mg %*	*mOsm/L*	*% body wt*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
W.M.	M	49	76.3	1.92	205/135	III	14	296	15.3	137	3.5	33.5	45.6
M.S.	M	49	71.8	1.83	225/125	II	22	281	13.8	141	3.6	41.3	49.0
V.W.	F	32	65.0	1.72	255/150	II	13	288	13.1	144	4.1	37.1	30.9
M.D.	F	36	70.5	1.72	195/125	II	13	288	13.8	142	4.3	33.1	29.9
R.D.	F	48	50.8	1.58	195/110	II	17	286	14.7	147	3.7	38.0	32.6
H.L.	F	44	65.4	1.61	220/115	II	9	288	14.0	144	4.2	33.8	28.0
M.S.	F	55	67.7	1.65	245/130	II	13	290	14.7	138	3.8	29.5	27.7
J.W.	M	46	83.2	2.00	205/110	II	14	291	14.6	141	3.6	45.1	44.1
M.H.	F	36	85.9	2.04	180/110	I	15	286	12.4	142	4.1	39.3	35.7
A.L.	M	50	67.8	1.77	235/145	III	17	283	16.2	141	3.9	41.5	42.1
D.C.	M	58	86.3	1.95	195/125	III	19	288	14.0	144	4.7	38.1	38.2
M.M.	F	60	99.5	1.98	230/130	III	20	297	15.1	141	4.2	33.9	26.1
C.A.	F	34	104.5	2.10	180/125	I	10	288	11.3	146	3.9	26.5	25.4
M.D.	F	46	76.8	1.83	215/125	II	13	281	14.7	145	4.5	35.1	30.3
H.B.	F	47	55.3	1.55	185/110	II	18	283	14.7	142	4.4	39.9	36.9
B.C.	M	36	85.9	2.04	175/115	II	14	290	12.7	144	4.2	32.3	35.1
H.B.	M	35	74.1	1.95	180/125	II	19	287	14.3	140	4.5	36.0	43.2
R.M.	F	53	63.3	1.59	180/120	II	16	302	13.9	140	4.2	42.1	30.4
G.H.	M	54	79.3	1.94	175/110	I	16	296	14.7	141	3.8	45.7	33.7
M.M.	F	49	69.1	1.71	175/100	II	12	286	14.2	142	4.2	39.3	36.5
						Control							
J.D.	M	52	74.5	1.89	120/80	0	18	286	15.8	139	4.0	41.2	39.9
J.L.	M	48	70.4	1.88	105/70	0	8	285	14.3	137	4.5	41.8	52.9
M.D.	F	33	66.4	1.76	105/65	0	12	279	15.9	138	4.3	38.0	35.0
J.M.	F	34	70.9	1.76	120/80	0	10	280	13.8	140	3.9	36.3	38.2
S.R.	F	45	49.5	1.50	125/75	0	17	289	17.0	140	4.2	36.7	43.2
A.J.	F	43	66.3	1.65	100/65	0	8	288	16.1	143	3.8	37.8	34.7
G.H.	F	56	66.1	1.69	125/80	0	15	284	14.8	143	4.1	34.5	32.4
A.F.	M	49	81.8	1.91	125/75	0	13	297	16.5	146	4.8	37.7	38.3
M.E.	F	37	86.8	2.00	130/75	0	8	283	15.3	139	4.2	29.5	28.8
E.H.	M	48	66.6	1.75	125/80	0	19	289	14.0	140	4.3	42.0	40.5
S.P.	M	62	85.0	1.95	125/80	0	14	288	16.5	140	4.4	35.6	32.8
M.E.	F	62	95.5	2.03	130/80	0	18	292	13.5	140	4.5	32.6	26.7
E.V.	F	39	108.9	2.13	135/85	0	12	288	10.1	139	4.1	29.6	27.1
N.R.	F	43	73.6	1.83	135/85	0	7	283	14.6	140	3.9	30.7	32.6
M.M.	F	41	52.0	1.51	115/75	0	10	277	14.3	137	3.6	34.8	33.4
T.M.	M	33	88.2	2.06	120/75	0	17	287	13.5	145	4.5	35.2	42.9
H.T.	M	36	74.5	1.97	120/75	0	13	290	14.8	144	4.5	42.8	40.0
E.K.	F	49	66.8	1.61	100/60	0	12	287	14.2	140	4.5	33.9	30.4
H.D.	M	52	80.3	1.95	115/80	0	11	287	15.0	141	4.4	39.8	39.1
T.M.	F	49	70.5	1.70	130/70	0	14	286	14.1	138	3.5	32.6	32.3

* BSA = body surface area. BP = blood pressure. BUN = blood urea nitrogen. Osm. = osmolality. ECF = extracellular fluid volume. Na_s = serum sodium. K_s = serum potassium. Na_e = exchangeable sodium. K_e = exchangeable potassium.

TABLE II

"Renal" hypertension

Subject	Diagnosis*	Sex	Age	Body wt	BSA	BP	Fundi	BUN	Serum osm.	ECF ($S^{35}O_4$ space)	Na_s	K_s	Na_e/ body wt	K_e/ body wt
			yrs	*kg*	*m²*	*mm Hg*	*grade*	*mg %*	*mOsm/L*	*% body wt*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
J.W.	Pyelo.	M	50	78.9	1.95	205/125	III	33	296	16.0	140	5.0	39.6	34.7
V.C.	Pyelo.	F	43	53.6	1.51	185/125	II	13	287	13.0	139	3.9	32.4	40.8
A.G.	Pyelo.	F	32	68.6	1.73	170/110	II	16	283	13.8	141	4.4	39.4	32.4
G.A.	Pyelo.	F	46	70.0	1.81	180/115	I	21	287	12.5	143	3.9	37.5	32.7
B.R.	Pyelo.	F	38	63.6	1.72	255/180	II	17	292	12.2	134	3.6	30.6	34.6
G.R.	Polycyst.	M	24	87.6	2.10	150/110	II	14	287	13.0	139	3.9	32.4	40.8
M.C.	Polycyst.	F	45	65.8	1.61	195/135	II	23	283	16.2	141	4.3	42.9	32.8
M.M.	Neph.	F	28	60.1	1.71	200/125	II	13	288	15.6	144	4.0	33.1	31.0
G.T.	Neph.	M	46	82.0	1.95	175/115	III	20	288	15.4	140	4.8	35.9	49.1
A.G.	Neph.	F	55	73.1	1.73	195/125	II	20	289	14.1	143	4.0	31.0	30.9
							Control							
H.B.	Normal	M	52	80.3	1.95	115/80	0	11	287	15.0	141	4.4	39.8	39.1
M.M.	Normal	F	41	52.0	1.51	115/75	0	10	277	14.3	137	3.6	34.8	33.4
J.M.	Normal	F	34	70.9	1.76	120/80	0	10	280	13.8	140	3.9	36.3	38.2
N.R.	Normal	F	43	73.6	1.83	135/85	0	7	283	14.6	140	3.9	30.7	32.6
M.D.	Normal	F	33	66.4	1.76	105/65	0	12	279	15.9	138	5.3	38.0	35.0
H.G.	Normal	M	26	85.0	2.03	125/85	0	17	297	14.7	145	4.2	38.7	36.4
A.J.	Normal	F	43	66.3	1.65	100/65	0	8	288	16.1	143	3.8	37.8	34.7
F.W.	Normal	F	25	62.5	1.73	125/85	0	9	290	14.9	140	4.3	39.1	42.4
A.F.	Normal	M	49	81.8	1.91	125/75	0	13	297	16.5	146	4.8	37.7	38.3
T.M.	Normal	F	49	70.5	1.70	130/70	0	14	286	14.1	138	3.5	32.6	32.3

* Pyelo. = chronic pyelonephritis, bilateral. Polycyst. = polycystic kidney disease. Neph. = glomerulonephritis.

tricle with a prolonged decholin circulation time but without peripheral edema.

Radioactive sodium (Na^{24})[1] and radioactive potassium (K^{42})[1] were given orally in tracer doses of 80 and 125 μc respectively; 22 to 24 hours after the administration of the isotopes, serum and urine samples were obtained for the measurements of specific activity which were used for calculating exchangeable sodium (Na_e) and exchangeable potassium (K_e) (10, 11). Urine was also collected during the equilibration period for determination of sodium and potassium content and radioactivity excreted. After the first blood and urine samples were obtained, radiosulfate ($S^{35}O_4$)[2] in a dose of 50 μc was administered intravenously with a calibrated syringe. Twenty minutes after the injection, serum and urine were obtained for the determination of radiosulfate space (12).

For Na^{24} and K^{42} determinations, 4-ml aliquots of serum and urine were prepared in duplicate in Lusteroid test tubes and assayed in well-type sodium iodide and plastic phosphor[3] scintillation counters. By employing simultaneous equations the contribution of Na^{24} and K^{42} to the total activity of each sample was calculated (13). For $S^{35}O_4$ determinations, after the Na^{24} and K^{42} had decayed, serum proteins were precipitated with 10 per cent trichloroacetic acid and 1-ml aliquots of the supernatant were dried by infrared lamps in Pyrex planchets[4] with wafers of lens paper to ensure even distribution. Urine samples were similarly prepared without protein precipitation. S^{35} radioactivity was counted with a thin window Geiger-Mueller tube. Standards for the radiosulfate counting were made with an aliquot of the patient's serum obtained before radiosulfate administration to correct for the water content of the serum samples. Correction was made for the Gibbs-Donnan effect by multiplying the serum value by 0.9. Sodium and potassium were measured in urine and plasma with a lithium internal-standard flame photometer. Total solute concentration of the serum was determined by freezing point depression using a Fiske osmometer and sodium chloride standards of known osmolality. Results were expressed as serum osmolality without correcting for the contribution of serum proteins to the freezing point depression. Although serum water content was not directly determined, the "exchangeable" sodium and potassium values, calculated respectively from the specific activities of sodium and potassium in the serum and urine, were not significantly different.

Renal studies identical with those previously described (1) were also carried out in 5 of the patients with malignant hypertension. On the day of the test no medication was given. The subjects were studied in the morning in the postabsorptive state while lying supine. Urine was collected through an indwelling bladder

[1] Kindly supplied by Dr. A. K. Solomon, Dept. of Biophysics, Harvard Medical School, Boston, Mass.

[2] Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

[3] Obtained from Baird-Atomic, Cambridge, Mass.

[4] Obtained from Tracerlab, Boston, Mass.

TABLE III

Hypertension with mild heart failure *

Subject	Diagnosis	Sex	Age	Body wt	BSA	BP	VP	CT	Fundi	BUN	Serum osm.	ECF ($S^{35}O_4$ space)	Na_e	K_e	Na_e/ body wt	K_e/ body wt
			yrs	*kg*	m^2	*mm Hg*	*mm H₂O*	*sec*	*grade*	*mg %*	*mOsm/L*	*% body wt*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
R.C.	E.s. hyp.	M	50	64.4	1.70	235/135	55	25	II	21	289	20.2	141	3.5	50.9	36.0
E.W.	E.s. hyp.	F	44	50.0	1.47	230/135	75	30	II	19	285	26.1	142	3.5	51.0	34.0
E.H.	E.s. hyp.	M	51	63.8	1.81	170/115	60	21	II	16	285	20.9	141	3.7	49.4	36.0
S.L.	E.s. hyp.	F	48	39.5	1.35	245/145	85	23	II	19	271	20.2	134	4.0	45.0	35.6
R.W.	Pyelo.	M	63	88.6	2.11	185/125	75	28	III	30	303	16.8	142	3.7	46.8	31.5
M.S.	Pyelo.	F	55	45.7	1.40	255/135	155	31	III	90	325	18.1	142	5.0	50.5	34.4
E.G.	Neph.	M	58	61.4	1.72	230/130	100	26	II	20	280	17.0	139	4.0	51.5	34.7
							Control									
E.H.	Normal	M	48	66.6	1.75	125/80	60	12	0	19	289	14.0	140	4.3	42.0	40.5
S.R.	Normal	F	45	49.5	1.50	125/75	60	14	0	17	289	17.0	140	4.2	36.7	43.2
J.W.	Normal	M	49	65.9	1.81	115/75	45	10	0	11	293	16.2	140	3.7	42.6	34.2
A.L.	Normal	F	50	37.5	1.31	115/75	50	16	0	12	287	15.1	138	3.9	39.0	40.4
S.P.	Normal	M	62	85.0	1.95	125/80	85	17	0	14	288	16.5	140	4.4	35.6	32.8
M.A.	Normal	F	60	42.0	1.38	100/60	60	13	0	10	285	14.2	139	4.3	41.4	36.6
I.F.	Normal	M	54	61.4	1.71	130/75	70	15	0	14	291	15.3	142	4.6	44.6	39.8

* VP = venous pressure. CT = circulation time (decholin). Es. hyp. = essential hypertension. Pyelo. = chronic pyelonephritis, bilateral. Neph. = glomerulonephritis, subacute.

TABLE IV

Malignant hypertension *

Subject	Diagnosis	Sex	Age	Body wt	BSA	BP	VP	CT	Fundi	BUN	Serum osm.	ECF ($S^{35}O_4$ space)	Na_s	K_s	Na_e/ body wt	K_e/ body wt
			yrs	*kg*	m^2	*mm Hg*	*mm H_2O*	*sec*	*grade*	*mg%*	*mOsm/L*	*% body wt*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
M.D.	Ren. art. occl.	F	55	46.8	1.44	245/150	55	21	IV	22	285	21.1	139	3.4	48.5	35.1
J.H.	Es. hyp.	F	28	60.5	1.69	195/135	75	28	IV	44	294	22.6	140	3.3	53.1	38.8
D.D.	Es. hyp.	M	35	85.4	2.00	210/145	75	27	IV	27	303	20.7	138	5.0	51.0	42.3
R.N.	Es. hyp.	M	50	78.7	2.00	215/130	65	15	IV	22	320	16.6	144	4.2	32.9	30.2
I.M.	Neph.	F	34	85.9	2.04	240/150	40	16	IV	45	301	11.6	141	4.2	36.6	28.0
A.M.	Neph.	F	40	51.5	1.49	205/125	145	30	IV	20	293	24.1	140	4.8	67.0	29.9
E.H.	Pyelo.	M	40	75.3	2.02	210/135	45	18	IV	46	310	18.9	143	5.0	47.0	37.1
L.S.	Pyelo.	F	28	41.4	1.38	230/140	50	19	IV	35	345	14.0	142	4.4	42.9	43.3
R.M.	Pyelo.	M	45	69.8	1.73	205/135	70	25	IV	51	321	21.0	137	3.5	49.5	34.7
D.C.	Pyelo.	M	55	76.5	1.82	255/145	60	15	IV	20	288	14.2	143	4.7	38.3	34.2
							Control									
R.T.	Normal	F	56	47.2	1.50	115/75	50	13	0	17	288	16.0	142	4.4	36.9	43.1
F.W.	Normal	F	25	62.5	1.73	125/85	55	16	0	9	290	14.9	140	4.3	39.1	42.4
T.M.	Normal	M	33	88.2	2.06	120/75	30	14	0	17	287	13.5	145	4.5	35.2	42.9
H.D.	Normal	M	52	80.3	1.95	115/80	45	13	0	11	287	15.0	141	4.4	39.8	39.1
M.E.	Normal	F	37	86.8	2.00	130/75	60	12	0	8	283	15.3	139	4.2	29.5	28.8
M.M.	Normal	F	41	52.0	1.51	115/75	75	11	0	10	277	14.3	137	3.6	34.8	33.4
H.T.	Normal	M	36	74.5	1.97	120/75	65	18	0	13	290	14.8	144	4.5	42.8	40.0
A.A.	Normal	F	32	39.9	1.41	105/65	65	15	0	12	289	14.4	140	4.7	41.3	42.0
E.H.	Normal	M	48	66.6	1.75	125/80	60	12	0	19	289	14.0	140	4.3	42.0	40.5
S.D.	Normal	M	53	75.0	1.85	115/65	55	10	0	15	285	16.1	142	4.4	39.6	38.1

* Ren. art. occl. = renal artery occlusion. Neph. = glomerulonephritis, subacute. Pyelo. = chronic pyelonephritis, bilateral.

TABLE V

Sodium excretion in malignant hypertension

Subject	Procedure	C_{PAH}	C_{IN}	V	$U_{Na}V$	$U_{K}V$
		ml/min/1.73 m²		*ml/min*	*μEq/min*	
M.D.	Control	261	95	7.5	111	38
	5% NaCl (300 ml i.v.)	353	115	9.9	850	107
	Recovery	310	101	5.3	855	86
D.D.	Control	225	60	3.8	322	70
	5% NaCl (300 ml i.v.)	265	75	14.6	1,581	105
	Recovery	247	62	7.5	1,467	74
R.N.	Control	255	71	2.9	235	91
	5% NaCl (300 ml i.v.)	290	78	8.9	1,161	129
	Recovery	289	80	8.0	1,297	144
A.M.	Control	374	73	2.4	236	84
	5% NaCl (300 ml i.v.)	550	83	6.0	746	127
	Recovery	522	92	7.5	1,117	128
D.C.	Control	305	81	5.4	301	75
	5% NaCl (300 ml i.v.)	333	85	5.3	706	68
	Recovery	357	87	5.9	1,131	76

catheter. Following the "control" period which consisted of 3 to 4 urine collection periods of 10 to 15 minutes each, 300 ml of 5 per cent sodium chloride was administered intravenously at the rate of 10 to 12 ml per minute. At the end of the infusion a urine collection designated as "5 per cent NaCl" was made. Thereafter at least 3 additional urine collections were obtained in the "recovery" period at intervals of 15 minutes. The measurements of para-aminohippuric acid and inulin clearances, and concentrations of sodium and potassium in the urine and serum were made by methods previously reported (1).

RESULTS

The measurements of body fluid and electrolytes in hypertensive and control subjects are summarized in Tables I to V. The statistical analyses of these findings are given in Table VI.

Essential hypertension. Exchangeable sodium per kilogram of weight and the radiosulfate space expressed as percentage of body weight were not significantly different in the hypertensive and control groups. Although serum sodium and blood urea nitrogen were slightly but significantly elevated in the hypertensive as compared with the control group, serum osmolality was not significantly different in the two groups. Intracellular and extracellular exchangeable sodium, as calculated from the radiosulfate spaces and serum sodium values, also did not show a significant difference in the groups ($p > 0.4$). Serum potassium and total exchangeable potassium per kilogram of weight were not significantly different in the hypertensive and control groups.

Hypertension associated with bilateral renal disease. Serum sodium, serum osmolality, radiosulfate space and exchangeable sodium in the "renal hypertensive group" were not significantly different from the values obtained in the control group. Exchangeable potassium and serum potassium were also comparable in the groups.

Hypertension complicated by mild heart failure. Exchangeable sodium and radiosulfate spaces were significantly higher in the seven hypertensive subjects who had mild heart failure without clinically detectable peripheral edema than in the control group. The increase in exchangeable sodium averaged 9 mEq per kg weight or 22 per cent of the values in the control group, whereas the mean increase in the $S^{35}O_4$ space was 4.4 per cent of body weight, or 29 per cent of control values. However, serum sodium and osmolality did not differ significantly in the two groups. The mean serum potassium and exchangeable potassium were slightly reduced as compared with the control values. These differences in serum potassium were not statistically significant, but those in exchangeable potassium were significant at the 5 per cent level.

Malignant hypertension. The group with malignant hypertension as compared with the control group had significant elevations of exchangeable

TABLE VI

Mean data and statistical analyses

	Age	Body wt	BSA	Systolic BP	Diastolic BP	BUN	Serum osm.	ECF ($Sl^{24}O_4$ space)	Na_e	K_e	Na_e body wt	K_e body wt
	yrs	*kg*	m^2	*mm Hg*	*mm Hg*	*mg %*	*mOsm/L*	*% body wt*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
Control	45.9 ± 2.0*	74.7 ± 3.1	1.83 ± 0.04	120 ± 3	75 ± 2	12.8 ± 0.8	286 ± 1.0	14.7 ± 0.3	140 ± 0.6	4.2 ± 0.1	36.2 ± 0.9	36.1 ± 1.4
Es. hyp.	45.6 ± 1.9	74.9 ± 3.0	1.82 ± 0.04	202 ± 6	121 ± 3	15.2 ± 0.8	289 ± 1.2	14.1 ± 0.2	142 ± 0.6	4.1 ± 0.1	37.1 ± 1.1	35.1 ± 1.5
Mean difference	+0.3 ± 2.7	+0.2 ± 4.3	−0.01 ± 0.1	+82 ± 7	+46 + 12	+2.4 ± 1.1	+3 ± 1.6	−0.6 ± 0.4	+2 ± 0.8	−0.1	+0.9	−1.0 ± 2.1
p	>0.9	>0.9	>0.9	<0.01	<0.01	0.04	0.1	0.2	0.04	0.1	0.5	0.6
Control	39.5 ± 3.0	70.9 ± 3.1	1.78 ± 0.1	120 ± 3	76 ± 2	11.1 ± 0.9	286 ± 2	15.0 ± 0.3	141 ± 1	4.1 ± 0.1	36.6 ± 0.9	36.2 ± 1.0
Renal hyp.	40.7 ± 3.2	70.3 ± 3.3	1.78 ± 0.1	191 ± 9	126 ± 6	19.0 ± 1.9	288 ± 1	14.3 ± 0.5	141 ± 1	4.2 ± 0.1	35.8 ± 1.3	36.0 ± 1.8
Mean difference	+1.2 ± 4.4	−0.6 ± 4.5	0 ± 0.1	+71 ± 9	+50 ± 7	+7.9	+2 ± 2.5	−0.7 ± 0.5	0	+0.1 ± 0.2	−0.8 ± 1.6	−0.3 ± 2.1
p	0.8	0.9	0.9	<0.01	<0.01	0.5	0.2	0.2	>0.9	0.6	0.6	>0.9
Control	52.6 ± 2.4	58.3 ± 6.2	1.63 ± 0.1	119 ± 4	74 ± 2	13.8 ± 1.2	289 ± 1	15.5 ± 0.4	140 ± 1	4.2 ± 0.1	40.3 ± 1.2	38.2 ± 1.4
Hyp. & C.H.F.†	52.7 ± 2.4	59.1 ± 6.1	1.65 ± 0.1	221 ± 12	131 ± 4	30.7 ± 10.0	291 ± 7	19.9 ± 1.2	140 ± 1	3.9 ± 0.2	49.3 ± 0.9	34.6 ± 0.6
Mean difference	+0.1 ± 3.4	+0.8 ± 8.7	+0.02 ± 0.1	+102 ± 12	+57 ± 4	+16.9 ± 10	+2 ± 7	+4.4 ± 1.3	0 ± 1.2	−0.3 ± 0.2	9.0 ± 1.5	−3.6 ± 1.5
p	>0.9	>0.9	0.9	<0.01	<0.01	0.1	0.7	<0.01	0.8	0.2	<0.01	0.04
Control	41.3 ± 3.3	67.3 ± 5.3	1.77 ± 0.1	118 ± 2	75 ± 2	13.1 ± 1.2	286 ± 1	14.8 ± 0.3	141 ± 0.7	4.3 ± 0.1	38.1 ± 1.3	39.0 ± 1.5
Malig. hyp.	41.0 ± 3.2	67.2 ± 5.1	1.76 ± 0.1	221 ± 6	139 ± 3	33.2 ± 3.9	306 ± 6	18.5 ± 1.3	141 ± 0.7	4.2 ± 0.2	46.7 ± 3.1	35.4 ± 1.6
Mean difference	−0.3 ± 4.6	−0.1 ± 7.3	−0.01 ± 0.1	+102 ± 7	+64 ± 3	+20.1 ± 4.1	+20 ± 6	+3.7 ± 1.3	0 ± 1.0	−0.1 ± 0.2	+8.6 ± 3.3	−3.6 ± 2.2
p	>0.9	>0.9	>0.9	<0.01	<0.01	<0.01	<0.01	0.02	0.8	0.7	0.02	0.1

* Standard error.
† Hyp. & C.H.F. = hypertension with complicating congestive heart failure. Malig. hyp. = malignant hypertension.

sodium per kilogram of weight and of radiosulfate space expressed as percentage of body weight. The increases in the exchangeable sodium and the radiosulfate spaces were comparable and averaged 22 and 25 per cent above control values. These changes did not occur consistently in the group but appeared to occur only when there was also a prolonged decholin circulation time. Serum sodium was comparable in the normotensive and hypertensive groups, whereas serum osmolality was significantly elevated in the hypertensive group in association with nitrogen retention. Neither serum potassium nor exchangeable potassium was significantly different in the groups, although the mean exchangeable potassium was lower in the hypertensive than in the control group by 3.6 mEq per kg of weight.

An impairment in sodium excretory capacity, as indicated by the rate of sodium excretion following a rapid infusion of 300 ml of 5 per cent sodium chloride (Table V), was not demonstrable in five subjects with malignant hypertension, including three who had prolonged circulation times and increases in exchangeable sodium and extracellular fluid volume. The rate of sodium and potassium excretion in these five subjects was similar to that previously reported in uncomplicated hypertensive subjects and considerably higher than that found in normotensive subjects (1).

DISCUSSION

A number of conflicting reports have appeared concerning body fluid and electrolyte composition in arterial hypertension. Grollman and Shapiro have reported a slight but significant increase in the radiosulfate space in essential hypertension (14). In addition to an expanded extracellular fluid space, Ross has observed an increase in total exchangeable sodium in hypertensive subjects (15). In contrast to these observations, some other workers have found extracellular fluid volume and exchangeable sodium to be normal in arterial hypertension (16, 17). The current results suggest that the apparent discrepancies in these reports might be due to differences in selection and classification of the hypertensive subjects.

In the present study exchangeable sodium and extracellular fluid volume were not found to be significantly elevated in uncomplicated essential hy-

pertension. Exchangeable potassium also was not significantly altered in this form of hypertension. The latter finding is in sharp contrast to the marked reduction in exchangable potassium found in hypertension associated with primary aldosteronism (9). Hypertension associated with renal disease also appeared to be characterized by a normal body sodium, potassium and radiosulfate space. These observations indicate that the increased capacity of hypertensive subjects to excrete sodium is not due to an expanded extracellular fluid volume nor to an increase in body sodium as was previously considered (1, 2).

In contrast to uncomplicated hypertension, hypertension complicated by congestive heart failure was accompanied by an increase in both exchangeable sodium and radiosulfate space. These changes in body sodium and fluid volume were not clinically detectable in the early stages of heart failure, since they occurred without peripheral edema or weight gain during an unrestricted dietary salt intake. An elevated radiosulfate space has likewise been observed by Walser, Duffy and Griffin in mild heart failure without peripheral edema (16). The present and previous studies indicate that these alterations in body composition may also occur without a demonstrable impairment in sodium excretory capacity (18–20). In view of these observations it is possible that the increases in body sodium and extracellular fluid volume found by Ross in a group of subjects with severe hypertension might have been due to complicating subclinical heart failure.

An increase in body sodium and extracellular fluid volume also occurred in the malignant phase of hypertension without clinically detectable edema or an impairment in sodium excretory capacity. Since this accelerated form of hypertension was frequently complicated by severe vascular disease as well as by heart failure, it is not entirely clear whether the changes in body sodium and fluid volume were related to the hypertension itself or to the complications of the disease. However, it appears that body sodium may remain normal at the onset of malignant hypertension but may increase as the hypertension becomes complicated by severe cardiovascular disease, especially congestive heart failure. Complicating heart failure, which sometimes is clinically difficult to recognize in its early stages, may also account for the marked increase in aldosterone activity reported in severe and malignant cases of hypertension (5, 6). It is unlikely, however, that an augmented aldosterone activity per se is responsible for the changes in body fluids and electrolytes in malignant hypertension and congestive heart failure, since salt and water retention is usually greater whereas potassium depletion is considerably less in these conditions than in primary aldosteronism.

CONCLUSIONS

1. In uncomplicated essential and renal hypertension, exchangeable sodium (Na^{24} space), exchangeable potassium (K^{42} space), extracellular fluid volume ($S^{35}O_4$ space), and serum osmolality were not significantly different from the values obtained in control subjects.

2. In hypertension complicated by heart failure, even of a mild degree without clinically detectable peripheral edema, exchangeable sodium and radiosulfate space were significantly elevated, whereas exchangeable potassium was slightly reduced.

3. In malignant hypertension, exchangeable sodium and radiosulfate space were also significantly increased while exchangeable potassium was slightly reduced.

4. These body fluid and electrolyte patterns are significantly different from those found in the hypertension associated with primary aldosteronism.

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BODY FLUID AND ELECTROLYTE COMPOSITION IN ARTERIAL HYPERTENSION. II. STUDIES IN MINERALOCORTICOID HYPERTENSION *

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In a previous report, measurements of body fluid and electrolyte composition have been compared in normal subjects and patients with arterial hypertension (1). In this paper, similar studies are presented in a group of patients with primary hyperaldosteronism studied before and after surgical therapy and in a group of normal subjects given mineralocorticoid drugs. The results indicate that the findings in the patients with established primary hyperaldosteronism differ from those in patients with uncomplicated essential, renal and malignant hypertension.

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CLINICAL MATERIAL AND METHODS

Eleven patients with primary hyperaldosteronism and 11 normal control subjects who were matched according to age, sex, weight and surface area were included in the study. All patients had typical findings of primary hyperaldosteronism (2), which in 7 cases was associated with an adrenal adenoma and in 4 cases with adrenal hyperplasia. Every patient had sustained arterial hypertension with diastolic blood pressures in the group ranging from 110 to 140 mm Hg. Examination of the fundi revealed Grade IV hypertensive retinopathy in 1 patient, Grade III retinopathy in 2 patients and Grade II retinopathy in the remaining 8 patients. None of the patients had evidence of peripheral edema, clinical signs of con-

TABLE I

Primary hyperaldosteronism *

Subject	Sex	Age	Diagnosis	Body wt	BSA	BP	Fundi	Serum osm.	ECF ($S^{35}O_4$ space)	Na_e	K_e	Na_e/ body wt	K_e/ body wt
		yrs		*kg*	*m²*	*mm Hg*	*grade*	*mOsm/L*	*% body wt*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
S.W.	M	47	Rt. ad. adenoma	64.5	1.76	235/140	III	293	16.1	149	2.7	48.8	30.5
M.C.	F	37†	Rt. ad. adenoma	60.0	1.68	180/110	II	291		141	2.5	48.5	19.6
J.B.	F	48†	Bilat. ad. hyperplasia	57.8	1.57	210/125	IV	277	16.2	137	2.5	38.7	24.2
L.B.	F	31	Lt. ad. adenoma	56.8	1.56	200/130	II	296	15.8	145	2.9	40.2	23.4
M.A.	M	51	Lt. ad. hyperplasia	82.1	1.92	165/110	II	287	15.9	138	3.5	44.5	31.6
D.G.	F	35	Lt. ad. adenoma	53.4	1.58	190/120	II	295	16.5	140	2.6	44.1	28.5
J.M.	M	56†	Bilat. ad. hyperplasia	83.6	2.07	215/130	III	289	12.9	139	3.3	33.8	27.5
A.H.	F	46	Lt. ad. adenoma	51.8	1.48	210/125	II	294	17.0	148	2.8	42.7	27.3
C.B.	M	47†	Rt. ad. adenoma	68.2	1.84	220/135	II	290	20.2	142	3.5	38.5	29.3
J.S.	M	65	Bilat. ad. hyperplasia	69.1	1.72	205/120	II	291		144	3.0	46.9	
B.G.	F	33	Lt. ad. adenoma	61.8	1.68	210/120	II	292	21.0	145	2.6	42.4	22.5
						Control							
E.H.	M	48	Normal	66.6	1.75	125/80	0	289	14.0	140	4.3	42.0	40.5
S.R.	F	45	Normal	49.5	1.50	125/75	0	289		140	4.2	36.7	43.2
A.J.	F	43	Normal	66.3	1.65	100/65	0	288	16.1	143	3.8	37.8	34.7
M.D.	F	33	Normal	66.4	1.76	105/65	0	279	15.9	138	4.3	38.0	35.0
A.F.	M	49	Normal	81.8	1.91	125/75	0	297	16.5	146	4.8	37.7	38.3
M.M.	F	41	Normal	52.0	1.51	115/75	0	277	14.3	137	3.6	34.8	33.4
H.D.	M	52	Normal	80.3	1.95	115/80	0	287	15.0	141	4.4	39.8	39.1
S.M.	F	37	Normal	50.9	1.55	120/75	0	290	11.2	141	4.0	32.5	32.2
J.W.	M	49	Normal	66.4	1.81	110/65	0	293	13.8	140	3.7	40.5	54.5
S.W.	M	63	Normal	68.2	1.71	130/80	0	288		139	3.9	33.4	
E.K.	F	49	Normal	66.8	1.61	100/60	0	287	14.2	140	4.5	33.9	30.4

* BSA = body surface area. BP = blood pressure. ECF = extracellular fluid volume. Na_e = serum sodium. K_e = serum potassium. Na_e = exchangeable sodium. K_e = exchangeable potassium. Rt. ad. = right adrenal. Bilat. ad. = bilateral adrenal. Lt. ad. = left adrenal. Osm. = osmolarity.

† Values are means of 2 or more determinations.

gestive heart failure or nitrogen retention. Serum osmolality, radiosulfate space ($S^{35}O_4$), serum sodium (Na_s), serum potassium (K_s), total exchangeable sodium (Na_e) and total exchangeable potassium (K_e) were measured according to previously described methods (1). In 9 of the patients with aldosteronism, these studies were repeated within 6 weeks to 16 months following adrenal surgery for treatment of the aldosteronism and in 2 patients following preoperative supplementary potassium administration.

In addition, 8 normal subjects were treated with either 9-α-fluorohydrocortisone in daily oral doses of 1.0 mg or desoxycorticosterone acetate (DCA) in daily intramuscular doses of 20 mg. The dietary intake of sodium was controlled at 180 mEq of sodium daily, and potassium intake was unrestricted. Following a 1 week control period, treatment with either fluorohydrocortisone or DCA was instituted and continued for 15 to 21 days. Body weight was recorded daily and blood pressure 4 times daily. Serum Na, serum K, serum osmolality, radiosulfate space, exchangeable sodium and exchangeable potassium were measured during the control period and again at the end of the period of treatment. In Subjects J.W., T.M. and A.C., metabolic balance measurements were made in addition during a 21 day period of fluorohydrocortisone administration when dietary sodium was restricted to less than 9 mEq daily.

In 2 subjects with hyperaldosteronism and in 3 normal subjects simultaneous exchangeable sodium and potassium measurements and metabolic balance studies were carried out.

RESULTS

1. *Hyperaldosteronism*

The complete results are tabulated in Tables I, II, III and IV.

A. *Serum sodium.* Serum sodium was increased slightly, but not with statistical significance, in the patients with aldosteronism as compared with the control group, which was matched according to age, sex, weight and surface area. In the aldosterone group following operation, there was a slight reduction in serum sodium, averaging 4.1 mEq per L.

B. *Serum potassium.* Serum potassium was significantly reduced in the patients with aldosteronism, with a mean value of 2.9 mEq per L and a range of 2.5 to 3.5 mEq/L as compared with the control mean of 4.1 and range of 3.6 to 4.8 mEq/L. All patients with aldosteronism showed increases in serum potassium to the normal range following adrenal surgery.

C. *Serum osmolality.* Serum osmolality was slightly but not significantly increased in patients with aldosteronism, with a mean value of 290

TABLE II

Mean data and statistical analyses for Table I

	Age	Body wt	BSA	BP systolic	BP diastolic	Serum osm.	ECF (SnO_4 space)	Na_s	K_s	Na_e/body wt	K_e/body wt
	yrs	*kg*	m^2	*mm Hg*	*mm Hg*	*mOsm./L.*	*% body wt*	*mEq/L.*	*mEq/L.*	*mEq/kg*	*mEq/kg*
Control	46.3 ± 2.4	65.0 ± 3.2	1.70 ± 0.05	115 ± 3	72 ± 2	288 ± 1.7	14.6 ± 0.5	140 ± 0.7	4.1 ± 0.1	37.0 ± 0.9	38.1 ± 2.2
Hyperaldosteronism	45.1 ± 3.1	64.5 ± 3.2	1.71 ± 0.06	204 ± 6	124 ± 8	290 ± 1.6	16.8 ± 0.8	142 ± 1.2	2.9 ± 0.1	42.6 ± 1.4	26.4 ± 1.2
Mean difference	−1.2 ± 4.0	−0.5 ± 4.6	0.01	89 ± 7	52 ± 4	2 ± 2.3	2.2 ± 1.0	2 + 1.4	−1.2 ± 0.2	5.6 ± 1.7	−11.7 ± 2.5
p	0.8	>0.9	0.9	<0.01	<0.01	0.3	0.03	0.2	<0.01	<0.01	<0.01

mOsm per L versus a mean of 288 mOsm per L in the control group. There was no appreciable change in serum osmolality following adrenal surgery.

D. *Radiosulfate space.* The radiosulfate space in the aldosterone group was significantly increased, with a mean value of 10.8 L or 16.8 per cent body weight as against a mean of 9.7 L or 14.6 per cent body weight in the control group. Postoperatively, there was a mean reduction of 1.3 L in the eight patients who had these measurements carried out.

E. *Exchangeable sodium.* Exchangeable sodium in the aldosterone group ranged between 33.8 and 48.8 mEq per kg body weight as compared with 32.5 to 42.8 mEq per kg body weight in the control group. There was a mean increase in Na_e of 342 mEq in the aldosterone group, which was statistically significant. In the group of nine patients studied postoperatively, there was a mean reduction in exchangeable sodium of 201 mEq as compared with their preoperative values. Reductions were not confined to the early postoperative period but continued to occur in comparable amounts in the late postoperative period as well.

F. *Exchangeable potassium.* Exchangeable potassium was markedly reduced in all patients with aldosteronism, with values ranging from 19.6 to 31.6 mEq per kg body weight in contrast to the control range of 30.4 to 54.5. The mean reduction of exchangeable potassium for the group was 774 mEq. The two patients studied following potassium loading had increases in exchangeable potassium of 432 and 430 mEq. Postoperatively, there was a mean increase in exchangeable potassium of 663 mEq in the nine patients who were studied after adrenal surgery.

G. *Blood pressure.* All patients have now been followed for more than six months postoperatively. Four patients have had reduction in blood pressure to normal levels, six have had reductions of greater than 20/10 mm Hg but not to normal, and

TABLE III

Effects of therapy on body fluid and electrolyte composition in 9 subjects with primary hyperaldosteronism

Subject	Procedure	Body wt	BP	Serum osm.	ECF ($S^{35}O_4$ space)	Na_s	K_s	Na_e/ body wt	K_e/ body wt
		kg	*mm Hg*	*mOsm/L*	*L*	*mEq/L*	*mEq/L*	*mEq/kg*	*mEq/kg*
L.B.	Preop.	56.8	200/130	296	8.97	145	2.9	2,286	1,335
	Postop. 6 mos.	55.2	140/90	303	8.99	139	4.1	2,239	1,872
J.B.	Preop.	57.8	210/125	277	9.35	137	2.5	2,239	1,398
	Preop. K loading*	55.4	210/135	272	9.11	133	4.2	1,940	1,828
	Postop. 6 wks.	54.8	164/110	256	9.20	124	3.8	1,759	2,227
M.C.	Preop.	60.0	180/110	291		141	2.5	2,912	1,175
	Postop. 3 wks.	58.2	130/85	290		139	4.9	2,330	1,863
	Postop. 6 mos.	60.5	125/80	292		135	4.6	2,352	2,113
D.G.	Preop.	53.4	190/120	295	8.81	140	2.6	2,353	1,521
	Postop. 7 mos.	55.0	130/85	293	8.69	140	4.2	1,790	2,526
J.M.	Pre.op.	83.6	215/130	289	10.8	139	3.3	2,823	2,298
	Preop. K loading†	84.5	225/130	289	11.2	139	4.3	2,874	2,730
	Postop. 7 mos.	85.7	180/105	292	10.7	140	4.0	3,099	3,670
M.A.	Preop.	82.1	165/110	287	13.1	138	3.5	3,649	2,591
	Postop. 2 mos.	77.2	155/105	289	11.1	140	4.1	3,347	3,134
	Postop. 7 mos.	84.6	160/110			142	4.1	3,572	3,049
C.B.	Preop.	68.2	220/135	290	13.8	142	3.5	2,623	1,997
	Postop. 13 mos.	65.4	170/110	287	9.28	139	4.3	1,648	2,236
A.H.	Preop.	51.8	210/125	294	8.78	148	2.8	2,212	1,414
	Postop. 16 mos.	50.2	180/110	289	7.83	141	4.3	2,405	1,612
B.G.	Preop.	61.8	210/120	292	13.0	145	2.6	2,621	1,396
	Postop. 16 mos.	66.6	135/90	293	10.4	140	4.1	3,043	1,788

* Cumulative potassium intake for 8 days = 1,772 mEq.
† Cumulative potassium intake for 4 days = 957 mEq.

TABLE IV

The effects of DCA and 9-α-fluorohydrocortisone in 8 normal subjects during a daily sodium intake of 180 mEq

Subject	Sex	Age	Procedure	Body wt	BP	Serum osm.	ECF ($S^{35}O_4$ space)	Na_s	K_s	Na_e	K_e
				kg	*mm Hg*	*mOsm/L*	*L*	*mEq/L*	*mEq/L*	*mEq*	*mEq*
J.W.	M	49	Control	66.4	108/64	293	9.2	144	3.54	2,700	3,619
			Fluoro.*	68.2	116/70	294	11.1	148	3.29	3,363	3,674
F.W.	F	25	Control	62.5	124/84	290	9.31	140	4.26	2,442	2,651
			Fluoro.*	62.9	124/90	285	9.50	149	3.98	2,570	2,621
T.M.	M	33	Control	88.2	120/75	287	11.9	145	4.46	3,098	3,787
			Fluoro.*	90.0	140/90	291	12.6	145	4.01	3,519	3,478
H.T.	M	36	Control	74.5	118/76	290	11.0	144	4.54	3,185	2,978
			Fluoro.*	75.8	116/72	289	10.9	149	4.20	3,315	2,876
E.G.	M	24	Control	80.7	106/62	296	10.9	141	4.25	2,906	2,969
			DCA†	80.7	130/80	293	14.3	146	3.39	3,309	2,893
L.S.	M	23	Control	79.6	132/82	294	10.8	141	4.10	3,458	3,271
			DCA†	81.1	160/104	307	11.2	151	3.51	3,729	3,274
A.L.	M	34	Control	54.5	138/88	290	9.0	144	4.50	2,478	2,111
			Fluoro.*	53.6	160/108	290	11.2	144	4.08	2,705	1,916
EP	F	53	Control	58.6	132/78	288	9.40	141	5.24	1,903	1,755
			Fluoro.*	58.6	156/96	290	11.4	143	3.90	2,140	1,512

* 9-α-Fluorohydrocortisone, 1.0 mg/day orally for 15 days.
† Desoxycorticosterone acetate, 20 mg/day i.m. for 21 days.

one has had no reduction in blood pressure. The reduction in blood pressure was usually observed during the first postoperative month and occurred in patients with either adrenal cortical hyperplasia or adrenal cortical adenoma. In all instances, reductions in blood pressure were associated with increases in exchangeable potassuim. The changes in exchangeable sodium were variable however. In Subjects J.M., L.B., A.H. and B.G., postoperative reductions in blood pressure were not associated with reductions in exchangeable sodium, although some reduction in exchangeable sodium was observed in the other five individuals.

Neither of the two patients repleted with potassium preoperatively had a reduction in blood pressure.

2. Mineralocorticoid drug therapy

The complete results are tabulated in Table V. Five of the eight normal subjects treated with either DCA or fluorohydrocortisone had increases

TABLE V

The effects of 9-α-fluorohydrocortisone in 3 normal subjects during a daily sodium intake of 9 mEq

Subject	Sex	Age	Na intake	Procedure	Duration	Weight	BP	Net Δ Na balance
		yrs	*mEq/day*		*days*	*kg*	*mm Hg*	*mEq*
T.M.	M	33	180	Control	7	88.2	120/70	−20
			9	Fluoro.*	14	88.1	122/72	+8
A.L.	M	34	180	Control	7	54.5	138/88	−39
			9	Fluoro.*	14	54.7	136/84	+60
E.P.	F	43	180	Control	7	58.6	132/78	+18
			9	Fluoro.*	14	58.6	130/82	−3

* 9-α-Fluorohydrocortisone, 1.0 mg/day orally.

TABLE VI

Comparison of changes in body Na and K by metabolic balance and radioisotope space measurements

Subject	Period of observation	Metabolic balance Δ Na	Na_e	Metabolic balance Δ K	K_e
	days	*mEq*		*mEq*	
J.M.	17	−160	+59	+151	+138
E.B.	23	−86	+102	−2	+144
	14	−173	−350	+249	+358
A.L.	14	+274	+227		
L.S.	14	+376	+271		
E.G.	14	+383	+403		

in blood pressure of greater than 20/10 mm Hg while on a dietary sodium intake of 180 mEq per day, although no increase in blood pressure was observed in all three of these five subjects who were treated with fluorohydrocortisone when dietary sodium was restricted to less than 9 mEq per day. In each instance when blood pressure increased following mineralocorticoid administration, there was a definite increase in exchangeable sodium, ranging from 227 to 421 mEq, along with slight increases in the radiosulfate space and reductions in serum potassium. Body weight, serum osmolality and exchangeable potassium in these five subjects did not show consistent alterations.

3. *Simultaneous metabolic balance, exchangeable sodium and exchangeable potassium measurements*

The complete results are tabulated in Table VI. Changes in body sodium and potassium as measured by radioisotope dilution techniques were comparable with those observed using the metabolic balance method. The maximum difference between the values obtained by each method was 219 mEq for sodium and 146 mEq for potassium —less than 10 and 8 per cent, respectively, of the total exchangeable sodium and potassium values.

DISCUSSION

The body fluid and electrolyte composition of patients with primary hyperaldosteronism differs from that observed in patients with other types of hypertension studied in this laboratory. Although, as in nonedematous patients with malignant hypertension or hypertension complicated by heart failure, there is a significant increase in exchangeable sodium and extracellular fluid volume, the severe degree of body potassium depletion present in this group has not been observed in any other untreated hypertensive state.

The relationship of the changes in body fluids and electrolytes to the pathogenesis of "mineralocorticoid" hypertension remains obscure. It is unlikely that absolute changes in extracellular fluid volume or the body content of sodium and potassium can be used to explain the development of the hypertension. With respect to extracellular fluid volume and exchangeable sodium, although statistically significant elevations of these measurements were present in the aldosterone group, many of the values recorded appeared to be within the normal range, and postoperative reductions in blood pressure following adrenal surgery in five of eight patients were not associated with reductions in extracellular fluid volume or exchangeable sodium. With respect to body potassium, the exchangeable potassium was reduced in every case preoperatively and increased to normal levels in all subjects studied following adrenal surgery. However, preoperative dietary repletion of exchangeable potassium toward normal was not associated with a reduction in blood pressure, nor was repletion of body potassium following surgery always associated with a reduction in blood pressure. In some instances administration of "steroidal antagonist" compounds for brief periods to these patients (or to individuals pretreated with mineralocorticoid drugs) caused potassium retention without necessarily affecting the blood pressure (3). An indirect effect of potassium depletion on the development of the hypertension through such factors as the production of potassium depletion nephropathy cannot, however, be excluded. Likewise, although the hypertension may not have been related to absolute changes in exchangeable sodium and potassium, local electrolyte redistribution cannot be ruled out by the techniques employed. Such shifts may play an important role in the development of the hypertension.

Similar to the observations in experimentally-induced mineralocorticoid hypertension of animals (4, 5), blood pressure elevation in the normotensive subjects treated with mineralocorticoid drugs is prevented when the dietary intake of sodium is restricted. However, mild elevation in blood

pressure is observed in some of the subjects when a liberal dietary intake of sodium is administered (6). As in the patients with primary hyperaldosteronism, it would appear that the changes in blood pressure with mineralocorticoid drugs may not be explained by mere absolute changes in exchangeable sodium, potassium or extracellular fluid volume. Not all subjects exhibiting increases in exchangeable sodium and extracellular fluid volume with mineralocorticoid drugs had increases in blood pressure, and the changes in exchangeable potassium with treatment were generally small and variable. Treatment with steroidal antagonist compounds likewise did not necessarily reduce the blood pressure to control levels (3) even though body sodium balance did return to or toward control levels. The differences in the severity of the hypertension and the degree of potassium depletion in the drug-treated group as compared with the patients with primary aldosteronism might be a result of the differences in duration of mineralocorticoid excess, the unphysiological doses of mineralocorticoid drugs that were used in this study, or possible differences in vascular reactivity between the two groups.

The close correlation between the radioisotope dilution and metabolic balance measurements is in agreement with previous reports (7–9), and again points to the usefulness of these techniques in evaluating changes in body electrolytes, particularly over long periods of time when metabolic balance measurements become impractical and difficult. From this study as well as others previously reported (9, 10), it would appear that measurements of exchangeable potassium are useful in determining the degree of potassium depletion present and yield information not obtainable from serum potassium determinations. Severe degrees of potassium depletion were present in all subjects with aldosteronism even though serum potassium levels were at times only slightly reduced. In the subjects treated with mineralocorticoid drugs, marked changes in serum potassium occurred at times without appreciable change in exchangeable potassium.

SUMMARY

1. Values of exchangeable sodium, potassium and extracellular fluid volume have been compared in 11 patients with primary aldosteronism and matched normal controls.

2. The results indicate that primary aldosteronism, unlike other uncomplicated hypertensive states, is characterized by a combination of slight increases in exchangeable sodium and extracellular fluid volume and marked reductions in exchangeable potassium.

3. Corrective adrenal surgery has resulted in restoration of exchangeable potassium to predicted normal levels and in some instances in reduction of exchangeable sodium and extracellular fluid volume.

4. Blood pressure elevation in normal subjects treated with mineralocorticoid drugs is associated with significant increases in exchangeable sodium and extracellular fluid volume.

5. The hypertension associated with mineralocorticoid excess does not appear to be directly related to absolute changes in body electrolytes.

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THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO THE PHOSPHOLIPIDS OF HUMAN LEUKEMIC LEUKOCYTES AND PLATELETS *

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The phospholipid composition of human platelets and erythrocytes has been reported by several investigators (1–4) and the metabolism of phospholipids in human blood has been studied in some detail (5–9). Recent investigations of phospholipid metabolism of leukocytes have been reported (6, 10) but we are not aware of similar studies on platelets.

In this laboratory, investigation of the biochemical characteristics of leukemic leukocytes is being carried out, and as part of this program an examination of the phospholipid metabolism of these cells was undertaken. In this report are presented data on the phospholipid composition of platelets and leukocytes from leukemic patients, and the results of studies on the incorporation of P^{32}-labeled orthophosphate (P^{32}) into the phospholipids of these formed elements *in vivo* and *in vitro*.

The phospholipid composition was similar in leukocytes and platelets from chronic leukemia and in leukocytes from acute leukemia. The pattern of incorporation into leukocyte phospholipids of P^{32} administered therapeutically to patients with chronic myelocytic leukemia was studied, and it was found that rapid incorporation of P^{32} could be demonstrated in lecithin, phosphatidylethanolamine, phosphatidylserine, inositol phosphatide, and sphingomyelin. In the course of this work it was also found that P^{32} is rapidly incorporated into the phospholipids of platelets, but is incorporated only slowly into the phospholipids of erythrocytes.

Observations were made on the *in vitro* incorporation of P^{32} into the phospholipids of platelets and leukemic leukocytes. Active incorporation of P^{32} by all phospholipid components of both formed elements was demonstrated, with the greatest incorporation occurring in inositol phosphatide and a component believed to be phosphatidic acid.

MATERIALS AND METHODS

Patients studied. Two patients with acute myelocytic leukemia, 2 with chronic lymphocytic leukemia, and 9 with chronic myelocytic leukemia were utilized in this study. Seven of the patients had not been treated previously for their leukemia. Six of the patients, all with chronic myelocytic leukemia, had been treated previously by chemotherapy or with P^{32}, but were in relapse with leukocyte counts above 100,000 per mm^3 at the time of the study. There was no apparent difference in the results obtained from previously treated or untreated patients, although the series is too small to permit definite conclusions on this point.

Separation of the formed elements of the blood. Leukocytes were isolated from patients with acute or chronic myelocytic or lymphocytic leukemia with white blood cell counts greater than 100,000 per mm^3. Five hundred ml of blood was collected in a plastic platelet pack (Fenwal) containing 50 ml of 3 per cent ethylenediamine tetraacetic acid (USP) as anticoagulant. All subsequent operations were performed at 4° C. The blood was stored for 1.5 hours to permit sedimentation of the erythrocytes. The plasma layer containing platelets and leukocytes was removed by syphoning into a plastic transfer pack (Fenwal) and then centrifuged in silicone-coated (G.E. Drifilm) 40 ml centrifuge tubes at 180 G for 15 minutes to sediment the leukocytes. The leukocytes were washed 3 times with twice their volume of 0.85 per cent NaCl solution and each time were collected by centrifugation at 180 G for 15 minutes. Platelets were isolated by centrifuging the platelet-rich plasma at 2,500 G for 10 minutes and were washed 3 times with 0.85 per cent NaCl solution. Erythrocytes from the sedimented layer of the

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original plastic bag were collected by centrifugation at 180 G and were washed 3 times with twice their volume of 0.85 per cent NaCl solution. Platelet-poor plasma was obtained by recentrifuging plasma after the isolation of platelets twice at 2,500 G for 15 minutes.

The leukocyte preparations were contaminated with small numbers of erythrocytes and platelets (3 to 4 erythrocytes and 10 to 20 platelets per 100 leukocytes). There was little contamination of the platelet preparation as determined by microscopic study of the suspension stained supravitally with neutral red and Janus green. The erythrocyte preparation usually contained 25,000 to 50,000 platelets per mm^3. Platelet-poor plasma contained 10,000 to 20,000 platelets per mm^3. Platelet counts were done by the method of Brecher and Cronkite (11) using phase contrast microscopy, and erythrocyte and leukocyte counts were done with a standard counting chamber after appropriate dilution.

Preparation of phospholipid extracts. Phospholipid extracts of the above samples were made by adding freshly prepared cell suspensions or plasma slowly to 15 volumes of chloroform : methanol (1 : 1, vol/vol) at room temperature and then homogenizing the mixture in a Waring blender in an atmosphere of nitrogen for 2 minutes (12). The mixture was allowed to stand at room temperature for 15 minutes and then was filtered. The residue was re-extracted with the same volume of chloroform : methanol (1 : 1). In preliminary experiments a third extraction yielded 1.1 per cent of the P present in the first two, and for this reason only two extractions were used as a routine. The combined chloroform : methanol extracts were emulsified with an equal volume of water. The mixture was centrifuged and the aqueous layer discarded. In some experiments the water wash was repeated after restoring the organic layer to the original volume with methanol. The organic layer was distilled to dryness under reduced pressure in an atmosphere of nitrogen. The residue was extracted with a small volume of isoamyl alcohol : benzene (1 : 1, vol/vol) and the lipid solution was stored at $-20°$ C.

Column chromatography. Phospholipid extracts were chromatographed on columns of silicic acid by the method of Hanahan, Dittmer and Warashina (13). The solvent was removed in a stream of nitrogen from an aliquot of the extract containing 3 to 5 mg of P and the residue was dissolved in a few milliliters of chloroform : methanol (19 : 1, vol/vol). The solution was applied to a column 1 cm in diameter containing 5 g of silicic acid (Mallinckrodt, AR) which had been heated at 110° C for 18 hours prior to use; 90 to 95 per cent of the applied P was recovered by stepwise elution with chloroform : methanol (19 : 1, 7 : 1, 4 : 1, 3 : 2, and 1 : 4, vol/vol). Occasionally the phospholipid was applied to the column in chloroform and the development begun with chloroform followed by the chloroform : methanol mixtures in sequence. The flow rate was usually 10 to 15 ml per hour and 2 to 3 days were required for complete elution of the phospholipids. Fractions of 5 ml were collected and total P was determined on each tube by the method of Fiske and Subbarow (14) using sulfuric acid-hydrogen peroxide to digest the samples. The appropriate tubes were combined and distilled to dryness *in vacuo* in an atmosphere of nitrogen. The residue was dissolved in isoamyl alcohol : benzene, 1 : 1 and stored at $-20°$ C.

Analysis of fractions. The components of the fractions from the columns were identified by chromatography at 4° C on silicic acid-impregnated paper using 2,6-dimethyl-4-heptanone : glacial acetic acid : water (40 : 20 : 3) as solvent (15). The components were identified by their Rf values and staining reactions with: rhodamine 6G; ninhydrin; 2,4-dinitrophenylhydrazine (15); and the choline spot test (16). The Rf values given in the text are averages derived from many chromatograms. Migration of the components was compared with purified phospholipids obtained from brain and soybean by solvent fractionation and column chromatography (13, 17, 18). Phosphatidic acid was prepared from soybean lecithin purified on an aluminum oxide column (18) using carrot lecithinase and was isolated as the sodium salt (19). A pure sphingomyelin preparation was kindly supplied by Dr. G. V. Marinetti of the University of Rochester.

Further confirmation of the identity of the components of the fractions was obtained by examination of acid hydrolysis products for ethanolamine, serine, choline, and inositol using paper chromatography and the following solvent systems: propanol : water (4 : 1), butanol : acetic acid : water (4 : 1 : 5), and phenol : water (4 : 1). Hydrolysis products of the phosphoinositide and phosphatidic acid fractions were studied in more detail as outlined below. The fractions were also tested for sterols by the Lieberman-Burchard reaction and for carbohydrate by the Molisch test. No free amino acids were present as indicated by paper chromatography.

Degradation of phosphoinositide and phosphatidic acid. In order to obtain information regarding the chemical nature of the highly-labeled components of the phosphoinositide and phosphatidic acid fractions, these were subjected to mild hydrolysis and the P-containing components identified by paper chromatography and analyzed for radioactivity.

The fractions containing phosphoinositide were hydrolyzed in 2 N HCl at 100° C for 10 minutes (20); the resulting water-soluble P compounds were chromatographed on paper with methanol : formic acid : water (16 : 3 : 1), propanol : ammonia : water (5 : 4 : 1), and propanol : acetic acid : water (3 : 1 : 1) (20, 21). The P-containing compounds were located by the method of Wade and Morgan (22), and were cut from the paper and assayed for radioactivity. Autoradiograms of the paper chromatograms were also prepared.

In experiments to determine the labeled hydrolytic products of the component migrating as phosphatidic acid, carrier soybean phosphatidic acid was added because of the small amount of material in this fraction. Following column chromatography the phosphatidic acid fraction was subjected to mild alkaline hydrolysis at 37° C for 20 minutes (23) and the water-soluble P compounds

were chromatographed on paper and assayed for radioactivity as outlined above.

Quantitative paper chromatography. The quantity of P present in each phospholipid spot was determined by extracting each component from the paper with 1 N methanolic HCl (24) and estimating the total P in the extract by the method of Bartlett (25). Triplicate estimations were made for each component.

Radioactivity measurements. Autoradiograms were prepared from paper chromatograms of the intact phospholipids after staining with rhodamine 6G, and of the P-esters after staining by the method of Wade and Morgan (22). The radioactivity of whole phospholipid extracts was determined by pipetting an aliquot in a thin layer on a metal planchet. The radioactivity of the individual components on the paper chromatograms was determined by cutting out the spots and affixing the paper to a planchet for counting. Correction for self-absorption was made when necessary. A thin window gas-flow counter was used for all determinations of radioactivity. All samples were counted for a sufficient time to give a standard error of less than 2.5 per cent.

In vitro studies. Leukocytes and platelets were prepared as above except that sterile techniques were employed throughout. The cells were suspended in platelet-poor, sterile plasma containing streptomycin, 100 μg per ml, and penicillin, 100 U per ml, and were incubated with $Na_2HP^{32}O_4$ at 37° C, in a stoppered tube in an atmosphere of air, with shaking for an appropriate time. Leukocyte and platelet counts were performed on an aliquot of the suspension taken prior to incubation. At the end of the incubation period the vessels were chilled to 0° C and the cells collected by centrifugation. The sediment was washed 2 or 3 times with ice-cold 0.85 per cent NaCl solution and the phospholipid extract prepared as outlined above.

RESULTS

Column chromatography. Leukemic leukocytes were obtained from two patients with acute myelocytic leukemia, two with chronic lymphocytic leukemia and nine with chronic myelocytic leukemia. Nineteen column chromatographic analyses were done on leukocyte phospholipids from these patients. Four major peaks were present in the column chromatograms (Figure 1). In some columns a small fifth peak was obtained with the final solvent, chloroform : methanol, 1 : 4.

One to 5 per cent of the total P eluted from the column was present in fraction I which also contained an unidentified yellow pigment and sterols as indicated by a positive Lieberman-Burchard test. On paper chromatography of this fraction two components were present: one migrated just behind the solvent front and probably represented nonphospholipid material, and the other had the

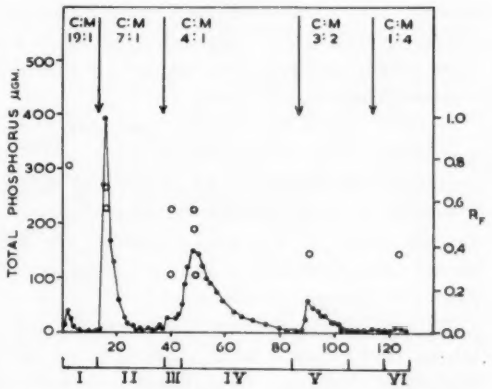

FIG. 1. CHROMATOGRAM OF HUMAN CHRONIC MYELOCYTIC LEUKEMIC LEUKOCYTE PHOSPHOLIPIDS (4.5 MG OF P) ON A 5 G SILICIC ACID COLUMN. The eluting solvents were mixtures of chloroform and methanol and are shown at the top of the figure. The numbers on the abscissa indicate the 5 ml fractions collected and beneath is shown the grouping of the tube contents into the six fractions discussed in the text. The open circles represent the Rf, indicated on the right ordinate, of the components present on paper chromatograms of each peak, as discussed in the text.

Rf (0.77) and staining characteristics of phosphatidic acid.

Fraction II contained 29 to 35 per cent of the P eluted from the column and on paper chromatography contained components behaving as phosphatidylethanolamine (Rf 0.67) and phosphatidylserine (Rf 0.57). The phosphatidylethanolamine contained some plasmalogen as indicated by reaction with 2,4-dinitrophenylhydrazine on the paper chromatograms. The acid hydrolysis products of this fraction contained ethanolamine and serine as demonstrated by paper chromatography. A positive Molisch test for carbohydrate was found with this fraction in some experiments.

Fraction III was the shoulder of the third peak and contained 2 to 4 per cent of the total P eluted. On paper chromatography it contained phosphatidylserine (Rf 0.57) and a phosphoinositide (Rf 0.28). The water-soluble products of acid hydrolysis of this peak contained serine and inositol.

Fraction IV was the remainder of the third peak and contained 53 to 59 per cent of the total P eluted. On paper chromatography of this peak large amounts of phosphatidylcholine (Rf 0.48) and small amounts of phosphoinositide (Rf 0.28) were

detected. On a few occasions a trace of phosphatidylserine (Rf 0.57) was also present. The water-soluble acid hydrolysis products of this peak contained choline and inositol.

Fraction V was usually the final peak eluted. It contained 7 to 10 per cent of the total P. A single component with the Rf (0.37) and staining properties of sphingomyelin was present on paper chromatograms of this peak. Rarely, the fifth fraction also contained a trace of phosphatidylserine and phosphatidylcholine.

Fraction VI when present contained only 0.5 to 1.0 per cent of the total P, and on paper chromatograms contained a single component behaving as sphingomyelin.

Quantitative estimation of the phospholipid components. Aliquots of the fractions obtained from column chromatograms were spotted on silicic acid-impregnated paper and after chromatography the P content of each component was determined in order to calculate the percentage of total P present. Data obtained in this manner from extracts of leukocytes from patients with chronic myelocytic leukemia are shown in Table I. Included also in Table I are data on the phospholipid composition of chronic myelocytic leukemic leukocytes obtained by direct paper chromatography of the whole phospholipid extract. It should be emphasized that with the paper chromatographic method used, only 80 to 90 per cent of the P applied to the paper can be recovered (24); our figures are subject to this limitation.

In vivo labeling of phospholipids by P^{32}. Patients with chronic myelocytic leukemia were given 3 mc of $Na_2HP^{32}O_4$ intravenously for therapy, and the incorporation of P^{32} into the blood phospholipids was followed at intervals. No radioactivity was detected in the phospholipids of the formed elements 2 hours after administration of P^{32}, but 18 hours later both leukocyte and platelet phospholipids were labeled, although no radioactivity was detected in the erythrocyte preparation. At 5 days the platelet and leukocyte preparations were strongly labeled and radioactivity had appeared in the red blood cell extracts. The slow appearance of radioactivity in the erythrocyte phospholipids compared with the other components of whole blood is emphasized in Table II wherein are presented the specific activities of the whole phospholipid extracts 8 days after therapy with P^{32}. In Figure 2 are presented autoradiograms of paper chromatograms of the phospholipid extracts of plasma and leukocytes obtained 5 days after administration of P^{32}. The principal components labeled in both plasma and leukocytes were phosphatidylethanolamine, phosphatidylcholine, and phosphoinositide, and faint labeling could be detected in sphingomyelin. In addition, leukocyte phosphatidylserine was faintly labeled. The labeling pattern was confirmed on phospholipid components separated by column chromatography prior to paper chromatography. A similar labeling pattern was found for platelet phospholipids from the patients.

In Table III are presented the specific activities of the phospholipid components from leukocytes obtained 6 days after administration of P^{32}. These data confirm the impressions obtained from the autoradiograms. No radioactivity was present in the component believed to be phosphatidic acid, and phosphatidylserine and sphingomyelin had approximately half the specific activity of the other components.

TABLE I

The phospholipid composition of chronic myelocytic leukemic leukocytes *

	Column fractions	Whole extract
	% total P	
Phosphatidic acid	3	4
Phosphatidylethanolamine	29	29
Phosphatidylserine	6	9
Phosphatidylcholine	46	42
Sphingomyelin	7	10
Phosphoinositide	9	6

* Quantitative paper chromatography was performed on whole phospholipid extracts and on fractions following column chromatography of the extracts. Figures for the column fractions are the average of two columns and for the whole cell extract are the average obtained from five extracts.

TABLE II

Specific activity of whole phospholipid extracts from chronic myelocytic leukemic blood obtained 8 days after the administration of 3 mc of P^{32}

	cpm/μg P
Plasma	21
Leukocyte	30
Platelet	25
Erythrocyte	9

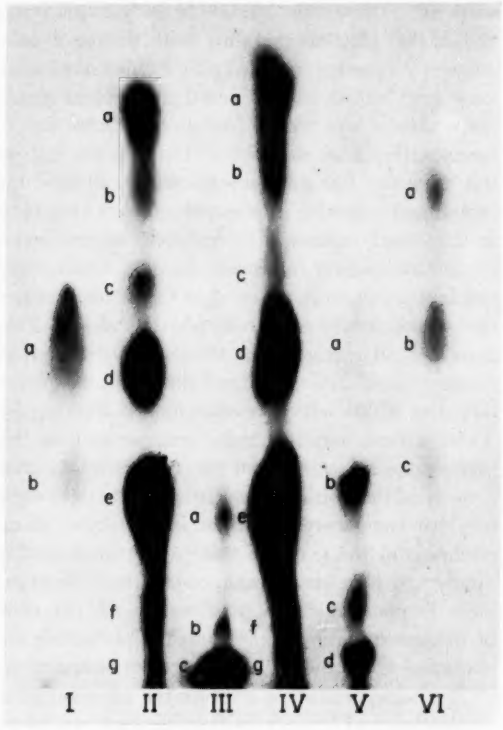

FIG. 2. AUTORADIOGRAMS OF PAPER CHROMATOGRAMS OF WHOLE PHOSPHOLIPID EXTRACTS OF BLOOD COMPONENTS EXPOSED TO P^{32} *IN VIVO* AND *IN VITRO*. Six μg of P was applied for each preparation; the film was exposed for 4 weeks. Lanes I and VI are from *in vivo* experiments and lanes II to V are from *in vitro* experiments. The bottom of the figure represents the starting line and the top the solvent front.

I. Extract of plasma from a patient with chronic myelocytic leukemia 5 days after P^{32} therapy (3 mc); a, phosphatidylcholine; b, sphingomyelin and phosphoinositide. These last two components were separated when stained with rhodamine 6G and examined under ultraviolet light, but on the autoradiograms they appear as a combined spot. Both were labeled when examined after separation by column chromatography. In addition, a faint spot with Rf of phosphatidylethanolamine could be seen on the original autoradiogram.

II. Extract of chronic myelocytic leukemic leukocytes after 6 hours' incubation of 7.5 ml of a suspension of 188,000 leukocytes and 36,000 platelets per mm^3 with 0.5 mc of $Na_2HP^{32}O_4$; a, phosphatidic acid; b, phosphatidylethanolamine; c, phosphatidylserine; d, phosphatidylcholine; e, phosphoinositide; f, unidentified component; g, inorganic P.

III. Plasma extract from II; a, phosphoinositide; b, unidentified component; c, inorganic P.

IV. Extract of platelets from a patient with chronic myelocytic leukemia after 6 hours' incubation of 7.5 ml of a suspension of 2,860,000 platelets per mm^3 with 0.5 mc of $Na_2HP^{32}O_4$. Identity of the components as in II. There was marked trailing of the highly-labeled phosphoinositide but on the original autoradiogram, spots f and g were clearly visible.

V. Plasma extract from IV; a, phosphatidylcholine; b, phosphoinositide; c, unidentified component; d, inorganic P.

VI. Extract of leukocytes from a patient with chronic myelocytic leukemia 5 days after P^{32} therapy (3 mc); a, phosphatidylethanolamine; b, phosphatidylcholine; c, phosphoinositide and sphingomyelin. In addition, a faint phosphatidylserine spot could be detected on the original autoradiogram.

TABLE III

Comparison of the incorporation of P^{32} into leukocyte phospholipids exposed to $Na_2HP^{32}O_4$ in vivo and in vitro *

	In vivo	*In vitro*
	cpm/μg P	*cpm/μg P*
Phosphatidic acid	0	3,300
Phosphatidylethanolamine	68	230
Phosphatidylserine	32	320
Phosphatidylcholine	70	450
Sphingomyelin	29	500
Phosphoinositide	83	10,600

* The data from the *in vivo* experiment were obtained from an extract of chronic myelocytic leukemic leukocytes obtained 6 days after the intravenous administration of 3 mc of $Na_2HP^{32}O_4$ to a patient. Paper chromatograms of the whole extract were used for determination of radioactivity and P. The reaction mixture for the *in vitro* experiment contained 0.5 mc of $Na_2HP^{32}O_4$, penicillin 750 U, and streptomycin 750 μg in 7.5 ml of a suspension of 188,000 leukocytes and 36,000 platelets per mm^3 from a patient with chronic myelocytic leukemia. The vessel was incubated for 6 hours at 37° C. The data presented were obtained by paper chromatography of the whole phospholipid extract.

In vitro studies of exchange of leukocyte and plasma phospholipids. It has been suggested that some plasma phospholipids are synthesized in the cells of the peripheral blood, and that plasma phospholipids are readily incorporated into the phospholipids of blood cells (7, 8, 10). In order to obtain evidence bearing on this possibility, experiments were performed using leukemic leukocytes and plasma containing phospholipids labeled with P^{32} *in vivo*. In one experiment a suspension of leukemic leukocytes labeled with P^{32} was sedimented at 2,500 G for 15 minutes and the packed cells were resuspended in 5 ml of unlabeled platelet-poor plasma. The platelet count of the final suspension was 90,000 per mm^3 and the packed cell volume was 11 per cent as determined in duplicate

by centrifuging an aliquot of the suspension in a capillary tube at 12,000 G for 10 minutes. The suspension was incubated at 37° C and at 0 and 6 hours 2-ml aliquots were chilled in ice and the cells and plasma separated by centrifugation at 2,500 G for 15 minutes. The plasma was recentrifuged at 2,500 G for 15 minutes, and the leukocytes were washed 3 times with 0.85 per cent NaCl solution. Phospholipid extracts were prepared from plasma and cells and the radioactivity present in each was determined. At 0 time plasma phospholipids contained 5 per cent of the radioactivity of the leukocyte phospholipids and at 6 hours contained 15 to 18 per cent of the radioactivity of the leukocyte phospholipids. In a converse study done in the same manner, a suspension of previously unlabeled leukemic leukocytes was incubated in 5 ml of labeled plasma. The packed cell volume of the suspension in this experiment was also 11 per cent. At both 0 and 6 hours the phospholipids of the cells contained 3 per cent of the radioactivity present in the phospholipids of the plasma.

Studies on incorporation of P^{32} into leukocyte and platelet phospholipids in vitro. When suspensions of leukemic leukocytes or platelets from normal or leukemic patients were incubated with $Na_2HP^{32}O_4$ and the phospholipids extracted and analyzed for radioactivity, it was found that there was rapid incorporation of radioactivity in all of the phospholipid components. The experimental details are given with Figure 2 and Table III. In a typical experiment the whole phospholipid extract contained 0.14 per cent of the total radioactivity added to the reaction mixture. No incorporation of P^{32} was found in the phospholipids of leukocytes or platelets of reaction mixtures sampled at 0 time. The incorporation of P^{32} into both platelet and leukocyte phospholipids increased with time, and reached a plateau at 4 to 6 hours. Incubation for 6 hours with P^{32} of a suspension in plasma of platelets damaged by freezing and thawing resulted in no incorporation of radioactivity into the phospholipids.

Since all of the leukocyte preparations were contaminated with some platelets, the possibility that the P^{32} incorporation might be entirely due to their presence was considered. Leukocytes and platelets from a patient with chronic myelocytic leukemia were employed. The incorporation of P^{32} into the phospholipids was compared in a suspension containing 522,000 leukocytes per mm^3 and 53,000 platelets per mm^3 with that in a suspension containing 210,000 platelets per mm^3 and very few leukocytes. Five-ml aliquots of these preparations were incubated in duplicate for 6 hours with 0.4 mc of $Na_2HP^{32}O_4$. At the end of this time the formed elements were collected by centrifugation and phospholipid extracts prepared in the usual manner. In order to ensure comparable extraction conditions for all vessels, carrier leukocytes were added after the incubation period to the vessels containing platelets alone. The phospholipid extract from the leukocyte preparation contained 0.87 mg P and that from the platelets after addition of carrier contained 0.94 mg P. Twice as much radioactivity was present in the phospholipid extract from the leukocytes as was present in the extract from the platelets. Assuming that the incorporation of P^{32} into the phospholipids of the platelets was proportional to the number of platelets in each vessel, the leukocytes were responsible for approximately 87 per cent of the incorporation of P^{32} into the phospholipids extracted from the leukocyte-platelet suspension.

The incorporation of P^{32} into the phospholipids of platelets and leukocytes from patients with leukemia is illustrated in Figure 2 by autoradiograms of paper chromatograms of phospholipid extracts. The pattern of incorporation of P^{32} into the phospholipids of normal platelets was similar to that found with platelets from patients with leukemia but no detailed quantitative comparison was made. No studies were done with normal leukocytes. The autoradiograms show that the phospholipids of the platelets were more intensely labeled than those of the leukocytes. All of the radioactive spots on the paper chromatograms could be identified except that labeled "f" in Figure 2. This component migrated with Rf 0.11 and was not detected with rhodamine 6G, ninhydrin, or the choline stain. Its identity is unknown but from its Rf it is possibly a nonlipid phosphorus compound (26, 27). Lysolecithin (4) and a phosphoinositide (28) have been reported to migrate with approximately this Rf but would have been detected with rhodamine 6G. Included in Figure 2 are autoradiograms of extracts of the plasma from the leukocyte and platelet suspensions incubated with P^{32}. The radioactivity was principally in the phosphoinositide component. The specific

activities of the plasma phospholipids in these *in vitro* experiments were less than 2 per cent of the specific activities of the leukocyte or platelet phospholipids.

In Table III are presented the specific activities of the components of the phospholipid extract from the leukemic leukocytes. The specific activities of the components migrating as phosphatidic acid and phosphoinositide were 6 to 40 times higher than those of the other components.

Because of their high specific activities the phosphoinositide and phosphatidic acid fractions were studied further in order to determine the chemical nature of the radioactive P present. The phosphoinositide fraction was subjected to acid hydrolysis and the water-soluble phosphate compounds released were separated by paper chromatography. The principal labeled compound migrated identically with authentic inositol monophosphate (California Corp. for Biochemical Research, Inc.). Some glycerophosphate and inorganic P were also present. The specific activity of the intact phosphoinositide was 390 cpm per μg P and that of the inositol monophosphate isolated from the hydrolysate was 410 cpm per μg P. These results indicate that the P^{32} in this component was in chemical combination with inositol and that the parent compound was a monophosphoinositide, probably phosphatidylinositol.

Attempts to identify the labeled component migrating as phosphatidic acid were carried out using the technique of co-chromatography (29). Relatively large amounts of unlabeled soybean phosphatidic acid were mixed with the labeled phosphatidic acid fraction and chromatographed on silicic acid-impregnated paper. The dried chromatogram was stained with rhodamine 6G to detect the phospholipid spots and an autoradiogram was prepared. The radioactivity was present in the phosphatidic acid spot and extended nearly, but not entirely, to the margin of the area stained with rhodamine 6G. As postulated by Hokin and Hokin (29) this result suggests that the unknown compound is similar to, but not identical with, the phosphatidic acid derived from soybeans. The difference may be due to differences in the fatty acids of the two compounds.

The phosphatidic acid fraction was also subjected to mild alkaline hydrolysis and the water-soluble phosphate compounds were separated by paper chromatography. The only labeled phosphate compound detected by direct counting or radioautography was glycerophosphate, suggesting that it was derived from labeled phosphatidic acid (23, 29, 30), rather than polyglycerophosphatide (31–34).

DISCUSSION

No significant qualitative difference was noted in the phospholipid composition of leukocytes obtained from patients with acute leukemia or chronic lymphocytic or myelocytic leukemia. The phospholipid composition of platelets was similar to that found for leukemic leukocytes. The analytical data agree essentially with those of others for platelets and erythrocytes (4). The presence of the component believed to be phosphatidic acid which constituted up to 4 per cent of the total phospholipids of both platelets and leukocytes is of particular interest because of its rapid incorporation of P^{32} *in vitro*. This component has been referred to as phosphatidic acid because of its behavior on co-chromatography with soybean phosphatidic acid and the finding that only glycerophosphate is formed on mild alkaline hydrolysis of the component. Troup, Reed, Marinetti and Swisher (4) have reported the presence of a phospholipid component of extracts of platelets and erythrocytes which they identified as a mixture of polyglycerophosphatides, including phosphatidic acids. It is possible that some polyglycerophosphatides were present in the small quantity of material studied here but were not detected. Both phosphatidic acids and polyglycerophosphatides have been identified in animal tissues (23, 29, 31–35).

The ability of the formed elements of blood to synthesize lipids has been studied by James, Lovelock and Webb (7) and the biosynthesis of phospholipids by whole blood has been studied by Rowe (9). Others have concluded that the leukocyte is the major site for lipid synthesis in blood (10) and that mature erythrocytes are not capable of synthesizing lipids (6, 10). The studies reported here indicate that platelets and leukemic leukocytes synthesize phospholipids also. Pure platelet preparations containing 200,000 platelets per mm^3 incorporated half as much P^{32} into phospholipid as a leukemic leukocyte preparation containing 500,000 leukocytes and 50,000 platelets per mm^3. It

could be inferred that platelets are the major site of incorporation of P^{32} into phospholipids *in vitro* in whole blood containing 10,000 leukocytes and 250,000 platelets per mm^3.

It has been suggested that plasma phospholipids may be synthesized in cells of the peripheral blood and that phospholipids of plasma may be readily incorporated into the phospholipids of blood cells (7, 8, 10). In experiments in which unlabeled leukemic leukocytes were incubated with labeled plasma no significant transfer of labeled phospholipid from plasma to cells was observed, whereas in the converse experiment a small amount of phospholipid was transferred from previously labeled leukemic leukocytes to plasma. The phospholipid appearing in the plasma may have been actively transported from living cells, or may have been released from leukocytes or contaminating platelets which lysed during the incubation period. In the experiments in which leukocytes or platelets suspended in plasma were incubated with P^{32} *in vitro* the small amount of radioactivity present in the phospholipids extracted from plasma could most probably be accounted for by platelets remaining in the plasma after centrifugation. Thus no definite evidence was obtained which might relate the metabolism of phospholipids in leukemic leukocytes and plasma.

In the *in vitro* experiments P^{32} was incorporated into all the phospholipid components of the leukocytes and platelets. The highest specific activities were attained by the component believed to be phosphatidic acid and by a phosphoinositide which probably has the structure of phosphatidylinositol. These findings are similar to those of Dawson (36) using guinea pig brain and those of Hokin and Hokin (37, 38) using pigeon pancreas and guinea pig brain. Rowe (9) reported that cephalin was the most highly labeled phospholipid component in whole blood incubated with P^{32}. This author did not identify phosphatidic acid or phosphoinositide as components of the phospholipid extracts, and it is possible that these compounds were responsible for much of the radioactivity present.

There is a striking difference between the labeling pattern of the phospholipids of leukocytes and platelets from patients receiving P^{32} therapeutically and the labeling when the cells are incubated with P^{32} *in vitro*. Label could be detected in the characteristic *in vivo* pattern in the leukocytes and platelets of patients 18 hours after injection of P^{32}. The *in vitro* incubations were usually performed for only 6 hours but when the incubation was extended to 18 hours the difference in pattern persisted, indicating that duration of exposure of the cells to P^{32} does not account for this result. The difference may reflect the persistent high level of P^{32} *in vitro* which could permit detection of components present in small quantity but having a rapid turnover of P. Another possibility is that P^{32} is incorporated into younger cells *in vivo* and the pattern of phospholipid synthesis may be directed more toward structural phospholipids than toward those with other physiological functions in young cells.

Dawson (39) has reported that the pattern of incorporation of P^{32} into phospholipids was different in guinea pig liver and brain when the P^{32} was administered *in vivo* and when slices of these organs were incubated with P^{32} *in vitro*, and Marinetti and co-workers (26, 27) have reported differences in the labeling pattern of phospholipids from rat liver when the P^{32} was administered *in vivo* and when homogenates of rat liver were incubated with P^{32}.

The incorporation of P^{32} from $Na_2HP^{32}O_4$ into the phosphoinositide and phosphatidic acid components of leukocytes and platelets incubated *in vitro* probably represents, at least in part, exchange of the phosphate moiety of the molecule rather than true synthesis of new phospholipid. From studies on the effect of acetylcholine on the turnover of P^{32} in phospholipids of various organs, Hokin and Hokin (40–42) have postulated that the turnover is related to transport of material across the cell membrane. Perhaps the turnover of P^{32} in some phospholipid components of leukocytes and platelets is related to the same phenomenon. It is also possible that this finding is related to the postulated role of phosphatidic acid as an intermediate in phospholipid synthesis (43). In any case the results obtained with platelets are evidence that these formed elements have very active phospholipid metabolism which must be included in any consideration of platelet physiology or disease.

SUMMARY

1. The phospholipid composition of platelets and leukocytes from patients with acute and chronic leu-

kemia has been studied by a combination of column and paper chromatography. The phospholipid composition of these formed elements was similar and included phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, sphingomyelin, a phosphoinositide, probably phosphatidylinositol, and a component believed to be phosphatidic acid.

2. The incorporation of P^{32} into the leukocyte and platelet phospholipids of patients given $Na_2HP^{32}O_4$ therapeutically has been studied. Radioactivity was present in the phospholipids 18 hours after administration of P^{32} and was found principally in phosphatidylethanolamine, phosphatidylcholine, and the phosphoinositide.

3. The *in vitro* incorporation of P^{32} into the phospholipids of leukemic leukocytes and platelets was also studied. The major incorporation of radioactivity occurred in the phosphoinositide and a component believed to be phosphatidic acid, in contrast to the pattern of labeling found *in vivo*.

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STUDIES OF BONE OF PATIENTS WITH HYPERPARATHYDROIDISM BY MEANS OF PHOSPHATE EXCHANGE EXPERIMENTS *IN VITRO* AND QUANTITATIVE MICRORADIOGRAPHY *

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The history of the interpretation of osteitis fibrosa cystica generalisata as it was described in 1891 by von Recklinghausen (1) explains why the skeletal abnormalities were for so long considered to be the focal point of the clinical concept "hyperparathyroidism." During the 1930's, however, it was observed that many patients with hyperparathyroidism showed no bone abnormalities on either histological or roentgenological examination (2). Recent publications show that only in one-third of the patients with hyperparathyroidism can skeletal deviations be demonstrated roentgenologically (3). This is remarkable, since a direct effect of parathormone on the skeleton has been established. It was demonstrated by treating nephrectomized rats with parathyroid extract (4), and by implanting parathyroid tissue under the skull of the mouse (5). Finally, it was shown that a cell-free parathyroid extract has a direct effect on tissue cultures of the parietal bone of mouse embryos and this by a reinforcement of the osteoclastic bone resorption (6).

The discrepancy between the clinical data and the pathophysiology of hyperparathyroidism might be explained by the assumption that the accelerated bone destruction caused by the hormone of the parathyroid gland is compensated for by an increase in bone production. If this hypothesis is correct, on the average, the bone of patients with hyperparathyroidism would be younger than bone of normal individuals of the same age. Evidence which supports this assumption has been found in animal experiments by using qualitative microradiography (7).

Using autoradiography, Engfeldt and Hjertquist (8) showed in 1954 that radioactive labeled phosphate is taken up in bone from a surrounding fluid, especially in young structures, and such uptake occurs in the same regions *in vivo* as in dead bone *in vitro*. This observation suggests that the uptake of phosphate from a solution by dead bone *in vitro* could be used as a measure of the ratio between the amounts of young bone and old bone present in the sample. By young bone is meant newly-formed bone; by old bone is meant older osteons and fragments of older bone. Since the uptake of phosphate from a solution by dead bone under certain conditions is primarily an exchange reaction, labeling of the phosphate of the incubation medium is necessary to follow the uptake and release of phosphate from the bone.

A method (9) for a phosphate "exchange" experiment *in vitro* was modified for the purpose of the present study, in such a way as to make its application to a clinical problem possible. The word, "exchange," is placed between quotation marks because there is a slight loss of phosphate from the incubation medium under the conditions used in our experiments.

The use of microradiography makes it possible to measure the mineral content of extremely small bone samples (10). Because of the likelihood of a fixed relationship between the mineral content and the "age" of bone (see Discussion), the investigation of the mineral content of bone from hyperparathyroid patients is important.

The studies to be described in this paper were performed in order to examine whether the mean age of bone from patients with hyperparathyroidism, as determined by the phosphate exchange capacity *in vitro* and by estimation of the mineral content per volume by microradiography, is consistent with the hypothesis that increased bone formation occurs in bone from these patients. The present report comprises the study of the bone of 17 patients with hyperparathyroidism, of 17 control individuals, and of 5 patients with hypercalcemia not caused by hyperparathyroidism, by a

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phosphate exchange test *in vitro* and by quantitative microradiography. Clinical data on the 17 patients with hyperparathyroidism are given in Table I.

The influence of the age of the individual on the phosphate exchange of the bone was investigated in a great number of bone specimens taken postmortem.

EXPERIMENTAL METHODS AND RESULTS

I. *A phosphate "exchange" test with powdered bone in vitro*

A. Method

The following technique was used in our studies. The bone specimen was taken by bone biopsy from the iliac crest (11), powdered and stored in 96 per cent ethanol for 24 hours. Fat and water were extracted with four 1-hour treatments, one with 96 per cent alcohol, one with absolute alcohol, and two with ether. The bone powder was then dried *in vacuo* and passed through a sieve with a pore diameter of 100 μ. Bone too coarse to pass through the sieve was reground in a mortar and then sifted. Duplicate samples of this powder weighing 10 mg each were put into round-bottom test tubes, 2.5 cm in diameter, and 4 ml of an incubation medium was added. This was a 0.005 M solution of KH_2PO_4 and Na_2HPO_4 with a pH of 7.3, labeled with radioactive phosphate (1 ml contained 2.10^{-2} μc).

It should be noted that the phosphate concentration of the incubation medium (0.005 M) is higher than that of the blood plasma. Its total ion concentration, however, is lower than that of blood plasma.

The bone powder was incubated with the labeled phosphate solution for 24 hours in a Dubnoff apparatus at 37° C and shaken at a rate of 120 per minute and an excursion of 6 cm. After this incubation period, the bone powder and the fluid were separated by centrifugation (2,500 rpm, 1,300 × G), and the supernatant was isolated. The bone powder was washed with 10 ml of distilled water, centrifuged for 10 minutes, and the supernatant combined with the supernatant from the first centrifugation, giving 14 ml of fluid. The bone powder was dissolved by boiling in 2 ml of 3 N hydrochloric acid, after which 12 ml of distilled water was added. The radioactivity of both the fluid obtained from the phosphate solution and that from the bone powder was measured in a Geiger-Müller tube designed for liquid counting (Veall tube, 20th Century Elec., type M 6). The phosphate content of both solutions was then determined. The percentage of bone phosphate involved in the "exchange" reaction during 24 hours of incubation is calculated from these data.

The exact percentage is calculated as a percentage of the value found in a standard bone sample, which is included in each experiment. This last value will be referred to as the relative percentage. The following equation was used for the calculation:

$$x = \frac{bc}{daf} \times 100,$$

where x = relative "exchange" percentage, a = total phosphorus content of bone sample, b = phosphorus content of incubation solution, c = counts per minute of incubation solution, d = counts per minute of "bone," f = "exchange" percentage of standard bone powder.

Bone powder made in great quantity from autopsy material was used as the standard. It was stored dry at 0° C. A standard was used because variations in results caused by small variations have the same effect on the standard as on the bone being investigated. The use of a standard therefore reduces the experimental error. The "exchange percentage" of the standard averaged 26.6 per cent.

Evaluation of the experimental error. A series of experiments was performed in order to evaluate the size of the experimental error. It was found that the standard deviation of the variation between duplicate samples ranged between 4 and 5 for a mean of 100 per cent. No increase in this standard deviation was found when duplicate samples of bone were taken from different points of the ventral part of the iliac crest. The same standard deviation was found when 2.5 mg of bone powder was used.

Preliminary experiments gave the impression that the percentage of exchange is slightly influenced by the concentration of phosphate in the incubation medium and by the grain size of the bone powder (12). Therefore, great care was taken to standardize these variables. It was found that the percentage of exchange of the bone from controls was not influenced when the bone sample was taken shortly after death or 24 hours postmortem.

Incubation time. To choose an optimal incubation period, the "exchange" reaction was followed for a total of 96 hours in bone samples with various "exchange" percentages (Figure 1).

The curves show that the difference in "exchange" of the samples from a patient with myxedema and the calf from the standard shows practically no change after 24 hours, the percentages for the calf, the standard, and the myxedema patient after 24 hours of incubation being 90, 100 and 130, and after 96 hours, 113, 128 and 157, respectively. A 24 hour incubation period was therefore chosen for this method.[1] It seems probable that differences between bone from normal individuals and from patients with bone disease can be most easily distinguished by means of an "exchange" test when the reactions studied are those which take place at the surface of the crystal. It does not seem obvious that *in vitro* recrystallization in dead bone (9), which causes the last and very slow-moving phase of the "exchange," will differ to any appreciable extent in different kinds of bone, although this possibility must be considered. Therefore,

[1] The amount of bone powder available made it impossible to do more than one test in duplicate although we had to choose one standard incubation time.

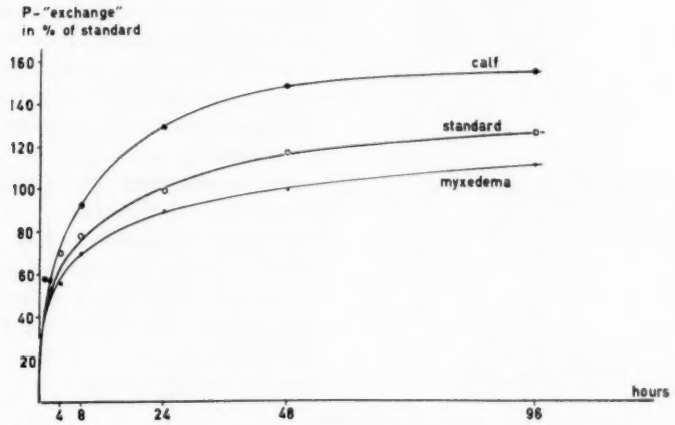

FIG. 1. THE RELATION BETWEEN INCUBATION TIME AND PHOSPHATE "EXCHANGE" IN BONE POWDER WITH A LOW, NORMAL, AND HIGH "EXCHANGE."

the choice of 24 hours as incubation period seems justified on theoretical grounds also.

B. Results

Effect of the age of the individual on the phosphate "exchange" of bone. Bone was obtained postmortem from a large group of individuals without bone disease and the percentage of phosphate "exchange" was measured. The results are given graphically in Figure 2. As can be seen from the graph, the phosphate "exchange" of the bone has an inverse correlation with the age of the individual. Because the shape of the curve which indicates the relationship between age and phosphate "exchange" was not easy to read from the points, it is difficult to supply a statistical calculation of the material. We have used an approximation, dividing the material into age groups which reflect the three periods of life: the growth period between 0 and 20 years, the adult period between 20 and 60 years, and the period of old age, 60 years and over.

TABLE I

Clinical data of 17 patients with hyperparathyroidism

Patient no.	Age	Sex	Duration*	Systems affected	Serum Ca	Serum P	Alk. phosph.	Urine Ca†	Renal function‡	X-rays‡	Bone histology‡	Surgery§
	yrs		*yrs*		*mg %*		*Bodansky U*	*mg/24 hrs*				
1	19	M	¼	Bone	16	2.2	16	500	−	+	−	+
2	19	M	3	Urogenital	14	2.1	4.9	260	−	−	−	+
3	19	M	½	Gastro intestinal	16	1.6	4.2	300	+	?	−	+
4	31	M	8	Urogenital	13	2.2	4.1	320	−	−	−	−
5	31	M	10	Urogenital	14.2	2.4	3.0	240	+	−	−	+
6	35	M	9	Urogenital	14	2.1	6.9	350	−	−	+	+
7	40	F	8	Urogenital	14	3	5.9	380	−	−	−	+
8	41	F	3	Urogenital	12	2	3	201	−	−	−	−
9	52	F	½	Bone and urogenital	12	2	11	500	+	+	−	+
10	59	F	4	Urogenital	14	2.3	5.7	600	−	−	−	+
11	59	F	6	Urogenital	12	3	8.0	270	+	−	−	+
12	60	F	6	Urogenital	11.7	2.5	3.4	280	−	−	−	+
13	61	M	6	Urogenital	11.5	2	5.5	110	+	−	−	+
14	61	M	4	Urogenital and bone	13.4	2.6	15	600	−	+	−	+
15	66	F	6	Gastro intestinal, bone and urogenital	13	5	21	100	+	+	+	+
16	73	M	3	Urogenital	14.9	4.4	7.3	300	+	+	−	+
17	74	F		Urogenital, epulis	12.5	2.1	7.5	70	+	−	−	+

* Expressed as an approximation of duration of illness.
† On low calcium diet.
‡ + Means abnormal; — means normal.
§ + Means successful treatment; − means unsuccessful treatment.

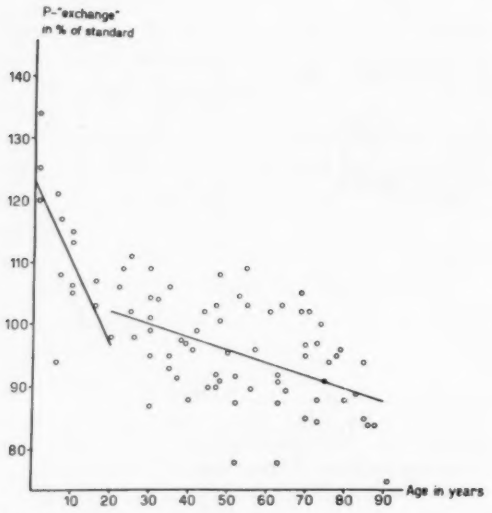

FIG. 2. RELATION BETWEEN AGE OF THE INDIVIDUAL AND PHOSPHATE "EXCHANGE" OF BONE IN MAN.

The linear regression coefficients were calculated for these three groups. Since the regression coefficient of the group between 20 and 60 years of age and that of 60 years and over did not differ significantly, in a second calculation of the material a division into two groups was used, one varying between 0 and 20 years, and the other comprising those over 20 years. These regression equations are:

$$y = 123 - 1.3 \times (0 \text{ to } 20 \text{ group})$$

$$y = 106 - 0.2 \times (20 \text{ to } 90 \text{ group}).$$

The regression coefficient, -1.3, differs significantly from zero ($p < 0.001$), as does that of -0.2 ($p < 0.01$). The regression of the group between 0 and 20 years of age and of those over 20 years differs significantly ($p = 0.001$). The correlation coefficients of both groups are, respectively, -0.73 ($p < 0.01$) and -0.49 ($p < 0.001$). Especially in very young individuals the influence of age is striking. The inverse correlation with age was also shown by an animal experiment. At the start of the experiment five animals from a group of 50 six-week-old male rats (Great Wistar) were killed. This was repeated at six-week intervals for six months, and thereafter every three months. The femora were cleaned and divided into fifths. The end parts were combined to form one sample and the central three-fifths formed the second sample. Bone powder was prepared from these samples and tested for phosphate "exchange." Five samples were used in each test and a mean value calculated. In Figure 3 the results are plotted against the age of the animals. An inverse correlation is seen between the phosphate "exchange" by the bone and the age of the test animals.

Hyperparathyroidism. The phosphate "exchange" capacity was studied in bone tissue from 17 patients suffering from hyperparathyroidism. The clinical data concerning these patients are presented in Table I. Material from 17 controls was also studied (Table II).

TABLE II

The phosphate "exchange" of bone from 17 patients with hyperparathyroidism and from 17 controls

Subject no.	Age	Sex	Phosphate exchange Abs.	Phosphate exchange Rel.
		Hyperparathyroidism		
	yrs		%	%
1	19	M	44.6	140.3
2	19	M	39.2	134.0
3	19	M	41.2	141.0
4	31	M	34.7	118.0
5	31	M	31.8	117.0
6	35	M	37.3	123.0
7	40	F	38.6	129.0
8	41	F	36.2	126.0
9	52	F	37.3	127.0
10	50	F	33.1	111.0
11	59	F	33.7	118.0
12	60	F	29.3	99.0
13	61	M	32.9	115.0
14	61	M	28.2	95.0
15	66	F	39.6	136.0
16	73	M	39.6	134.0
17	74	F	38.4	131.0
Mean	47			123.2
SD				13.2
		Controls		
1	12	M	32.0	108.8
2	22	M	29.7	106.0
3	23	M	30.3	108.0
4	25	M	28.7	102.0
5	30	F	27.9	95.0
6	35	M	29.0	106.0
7	41	F	26.2	96.0
8	42	F	30.1	99.5
9	47	F	23.0	92.0
10	47	M	29.4	103.0
11	47	M	26.6	90.0
12	48	F	29.6	100.5
13	52	M	29.4	93.0
14	57	F	26.2	96.5
15	69	M	30.1	102.5
16	74	F	31.7	100.9
17	87	M	26.4	84.0
Mean	45			90.0
SD				6.9

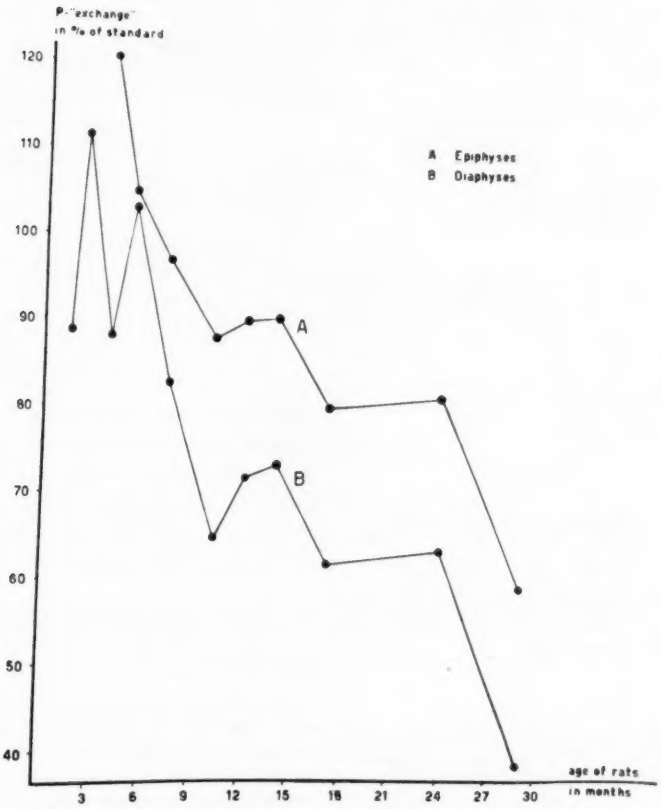

FIG. 3. RELATION BETWEEN AGE OF MALE RATS AND PHOSPHATE "EXCHANGE" OF BONE. Each point represents the mean of five animals. The 29 month age group consists of the two remaining animals.

The average "exchange" percentage in the hyperparathyroid group was 123.3 ± 13.2 per cent of the standard, while in the control group this was 99 ± 6.9 per cent. This difference is highly significant ($p < 0.001$). The data for both groups are shown graphically in Figure 4. Application of the χ^2 test shows that the results from Patients 10, 12, and 14 fall within the control group, while Patient 13 had a borderline value. The data for the remaining 13 patients can be considered as not belonging to the control group.

It was found that a small loss of phosphate from the incubation medium occurs during the incubation process (Table III). These data show that the mean amount of phosphate which is lost is larger in the group of patients (36 μg) than in the controls (7 μg). If we assume that the phosphate which is lost from the medium is shifted to the bone, it can be calculated that a considerable part of the increase of exchange in the bone of patients with hyperparathyroidism is due to an increased uptake of phosphate.

Samples of bone were taken for phosphate "exchange" measurements from two patients with hyperparathyroidism (Patients 1 and 15 in Table I) after successful surgical treatment of their disease. The results are given in Table IV. At the time of the second biopsy, Patient 1 was in good health for three years after the operation. Patient 15 died a year after the operation as a result of a kidney insufficiency. The biopsy was performed postmortem. The second samples showed for both patients a sharp drop in the phosphate "exchange" in comparison with the first samples.

A determination was also made of the phos-

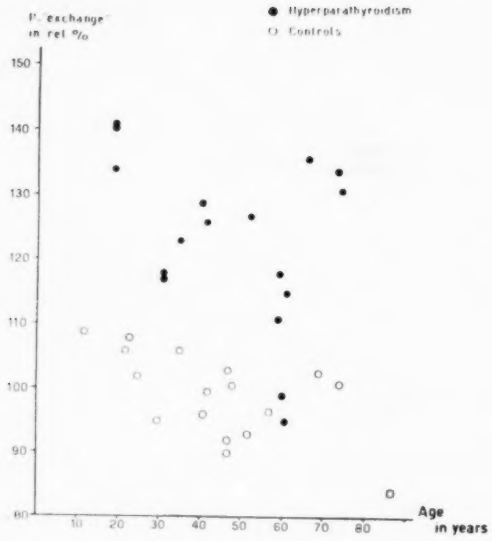

FIG. 4. COMPARISON OF PHOSPHATE "EXCHANGE" PERCENTAGE OF BONE FROM PATIENTS WITH HYPERPARATHYROIDISM WITH THAT OF CONTROLS. The exchange is expressed in a percentage of a standard.

TABLE III

Shift of phosphate from incubation medium to bone in the phosphate "exchange" tests for 17 patients with hyperparathyroidism and for 17 controls

Subject no.	Hyperparathyroidism Difference*	Controls Difference*
	μg	μg
1	−39	−15
2	−15	+ 5
3	−59	−35
4	− 5	−20
5	−28	−21
6	−55	−11
7	−33	+ 7
8	− 5	+14
9	−71	0
10	−75	−14
11	−29	−20
12	−21	−22
13	−40	+10
14	−26	+13
15	0	−27
16	−61	+15
17	−51	+15
Mean	−36	

* Difference means the total phosphate content of the solution incubated with bone from the subject, reduced by the total phosphate content of the solution incubated with standard bone. Where less than 10 mg of bone power was used, the difference was calculated from standard conditions.

TABLE IV

Phosphate "exchange" of bone from two patients with hyperparathyroidism before and after surgical treatment

No.	Remarks	Phosphate exchange: Before operation Abs.	Before operation Rel.	After operation Abs.	After operation Rel.
		%	%	%	%
1	Second biopsy 3 years after operation; patient healthy	44.6	140.3	29.7	106
15	Second biopsy 1 year after operation; patient died of uremia	39.6	136	23.8	87

phate "exchange" percentage of bone from five patients with hypercalcemia not caused by hyperparathyroidism. The results are given in Table V. In these five cases no increased percentage of exchange was found. All data are within the control group.

II. Quantitative microradiography

A. Method

Bone biopsies were obtained from patients and controls. The bone sample was held in 96 per cent alcohol for 24 hours and dehydrated with absolute alcohol and benzoyl benzoate. It was then imbedded in methyl methacrylate. A copper saw with an inner edge provided with diamond powder (13) was used to make slices 100 μ thick which were ground under water on ground-glass to about 70 μ. The width of slices was measured by microscope and ocular micrometer. An X-ray photograph was then made with an Enraf roentgen diffraction apparatus provided with a Philips copper anti-cathode tube with a focal size of 10 mm² and a mica beryllium window. The filter was 20 μ aluminum. The sample was photographed together with two aluminum

TABLE V

Phosphate "exchange" of bone from five patients with hypercalcemia not caused by hyperparathyroidism

Patient no.	Age	Diagnosis	Phosphate exchange: Abs.	Rel.
	yrs		%	%
1	20	Sarcoidosis	29.9	118
2	41	Vit. D intox.; idiopathic hypoparathyroidism	20.5	70 (Mean = 96)
3	51	Metastatic mammary cancer	27.6	93
4	60	Metastatic bronch. cancer?	29.3	107
5	63	Metastatic intest. cancer	25.3	94

TABLE VI

Experimental error of quantitative microradiography

Patient	Sex	Age	Site of the iliac crest biopsy*	Thickness of bone slice	Al eq.†
		yrs		μ	
Gast.	F	31	Left ventral	68	81.6 76.5 Mean = 78.9 76.0 SD‡ = 2.5 79.8 n‡ = 5 80.5
v.E.	M	52	Right ventral	90	92
			Right middle	80	91.2 Mean = 88.4
			Right dorsal	87.5	87.4 SD = 3.16
			Left ventral	90	86.7 n = 5
			Left dorsal	95	84.9
Zuid.	M	87	Right ventral	88	88.1
			Right dorsal	88.5	90.5 Mean = 90
			Left ventral	86.4	87.6 SD = 3.35
			Left middle	81	95.6 n = 5
			Left dorsal	91	88
Herm.	F	47	Right ventral	94	83
			Right middle	91	91.1 Mean = 88.7
			Right dorsal	81	89.5 SD = 3.20
			Left ventral	92	89.3 n = 5
			Left dorsal	87	90.4

* Ventral = 1½ cm dorsal of the anterior superior iliac spine; middle = 2½ cm dorsal of the anterior superior iliac spine; dorsal = 3½ cm dorsal of the anterior superior iliac spine.
† Equivalent of 100 μ bone expressed in microns of aluminum (sp gr of aluminum = 2.57).
‡ SD = standard deviation; n = number of observations.

step wedges having 10 steps each, built up of aluminum foil to a thickness of 10 μ and weighing 2.57 mg per cm² at that thickness. The photographic material was Kodak 649-0 and Kodak M.R. plates. The window of the roentgen tube was set at 25 cm from the preparation. The pictures were made with 20 kv and 25 ma. Exposure times were chosen so that the density, D ($D = \log I_0/I$), was about 0.4.

After development of the films microphotometric measurements were made with a self-registering photometer. A light beam with a diameter of 20 μ was used. The galvanometer readings from the X-ray pictures of the bone were converted into equivalent microns of aluminum by means of a curve based on the density caused by the aluminum step wedges. An average with a standard deviation was then calculated from all measurements made on the film, all averages being calculated for a bone thickness of 100 μ.

Evaluation of the experimental error. To estimate the size of the experimental error, a single bone sample was measured five times. The results are shown in Table VI. For an average value of 78.9 μ aluminum for 100 μ bone, a standard deviation of 2.5 was found. Next, from each of three patients, five biopsy samples were taken postmortem, three from the left iliac crest and two from the right iliac crest or vice versa. The biopsy samples were taken from an area 1 to 3 cm dorsal to the anterior superior iliac spine. The results obtained from this material are also shown in Table VI. The data indicate that small variations in the location on the iliac crest where the biopsies were done do not affect the experimental results. It should be noted that the patients here concerned did not have hyperparathyroidism.

It was found that the results obtained were independent of the thickness of the slices. The plastic embedding material, which caused only 1.2 per cent of the absorption of the X-rays in the bone, has hardly any effect on the final result.

B. Results

Bone tissue from the same 17 patients with hyperparathyroidism and 17 controls described above (Table I) was investigated. The results of the investigation of the bone of the patients and controls are given in Table VII. These data show that there is a highly significant difference between the mineral content of bone from normal individuals and from hyperparathyroid patients (equivalent for 100 μ bone: 84.6 ± 3.3 versus 74 ± 4; $p < 0.001$). The data for the individuals from both groups are shown graphically in Figure 5. The χ^2 criterion showed that the data from Patients 12, 13 and 14 did not significantly differ from the data found in the control group. The

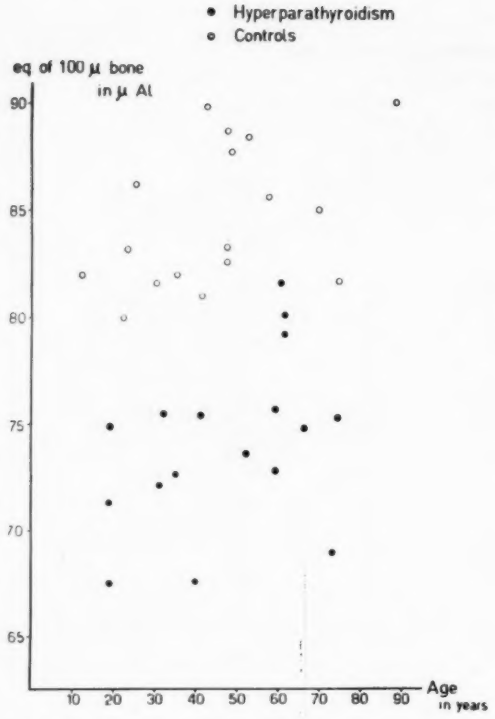

FIG. 5. THE MINERAL CONTENT OF BONE FROM 17 PATIENTS WITH HYPERPARATHYROIDISM COMPARED WITH THAT OF CONTROLS. The mineral content is estimated by quantitative microradiography and expressed in the thickness of aluminum foil that gives the same X-ray absorption as 100 μ of bone.

data from the remaining 14 patients can be considered as not belonging to those of the control group.

From the large number of measurements made at different points on each bone sample ($\pm$ 284 per sample), the standard deviation from the average found in that sample was calculated (Table VI, column 5). The average standard deviation for 17 patients with hyperparathyroidism was 9.2, and for the 17 controls, 7.6. This difference is significant at the $p < 0.05$ level.

Figure 6 shows all the measurements made on bone of hyperparathyroid patients and all those on bone from controls, divided into groups of 5 μ aluminum equivalent. The frequency distribution of the groups is shown graphically. The curve for the data from hyperparathyroid patients has a sharp maximum and is shifted to the left in comparison with the curve for the controls.

Samples from two patients (nos. 1 and 15 in Table VII) were measured for mineral content after the patients had recovered from their hyperparathyroidism following the removal of an adenoma of the parathyroid gland. The data from before and after treatment are given in Table VIII.

TABLE VII

Mineral content of bone from 17 patients with hyperparathyroidism and from 17 controls *

Subject no.	Age	Sex	Mean	SD	No. of photometric measurements
		Hyperparathyroidism			
	yrs				
1	19	M	67.3	6.5	465
2	19	M	71.2	13.3	168
3	19	M	74.9	8.8	142
4	31	M	72.1	8.2	217
5	31	M	75.5	5.3	156
6	35	M	72.6	12.6	642
7	40	F	67.5	11.0	297
8	41	F	75.4	10.4	472
9	52	F	73.6	6.7	297
10	59	F	72.8	12.1	194
11	59	F	74.7	7.5	147
12	60	F	81.6	3.7	97
13	61	M	79.6	13.7	224
14	61	M	80.1	7.6	223
15	66	F	74.8	10.5	562
16	73	M	68.9	9.2	245
17	74	F	75.3	10.1	200
Group mean	47		74	9.2	279
Group SD			4		
		Controls			
1	12	M	81.5	8.9	203
2	22	M	80	7.0	184
3	23	M	83.2	7.3	375
4	25	M	86.2	6.1	329
5	30	F	81.6	7.1	303
6	35	M	82.0	9.5	187
7	41	F	81.0	7.2	279
8	42	F	89.8	8.5	217
9	47	F	83.3	9.0	186
10	27	M	82.6	7.4	332
11	47	M	88.7	4.2	251
12	48	F	87.7	8.2	859
13	52	M	88.4	4.1	117
14	57	F	85.6	9.0	407
15	69	M	85.0	7.7	310
16	74	F	81.7	8.0	274
17	87	M	90.0	4.1	91
Group mean	45		84.6	7.6	288
Group SD			3.3		

* Values expressed as indicated in legend of Figure 5.

TABLE VIII

The mineral content of the bone of two patients with hyperparathyroidism before and after surgical treatment *

No.	Remarks	Mineral content	
		Pre-op.	Post-op.
1	Second biopsy 3 years after operation; patient healthy	67.3	80
15	Second biopsy 1 year after operation; patient died of uremia	74.8	78.2

* Mineral content expressed as indicated in legend of Figure 4.

The bone from both patients had a higher mineral content after the operation, but only in the first case was the difference statistically significant.

Bone samples from five patients with hypercalcemia not caused by hyperparathyroidism were analyzed for mineral content. The results are shown in Table IX. One of these patients, with hypoparathyroidism and vitamin D intoxication, had a low mineral content, while the other four patients revealed normal values.

TABLE IX

The mineral content of the bone of five patients with hypercalcemia not caused by hyperparathyroidism *

Patient no.	Age	Diagnosis	Mineral content
	yrs		
1	20	Sarcoidosis	79.5
2	41	Vit. D intox.; idiopathic hypoparathyroidism	74.3
3	51	Metastatic mammary cancer	84.7
4	60	Metastatic bronch. cancer?	88.5
5	63	Metastatic intest. cancer	83.5
		Mean	82

* Mineral content is expressed as indicated in legend of Figure 4.

DISCUSSION

It has been demonstrated that human bone from young individuals contains more water and less minerals than does bone from old individuals (14, 15). Tracer studies with intact experimental animals *in vivo* have established that the skeleton of young individuals shows greater uptake of bone-

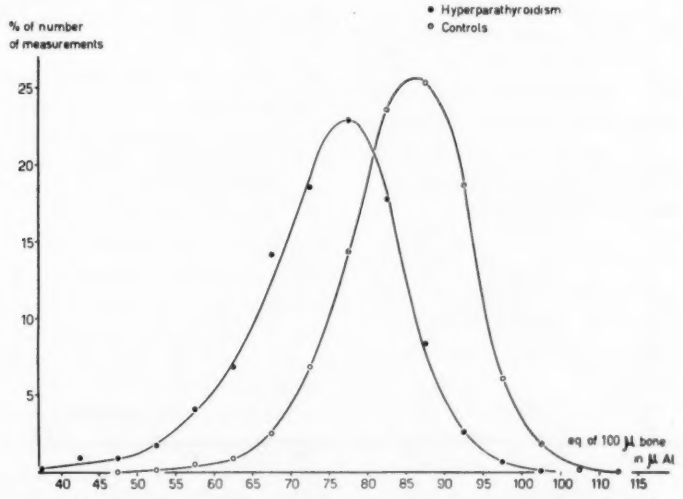

FIG. 6. DISTRIBUTION OF THE VALUES OF THE MINERAL CONTENT OF BONE IN 4,740 MEASUREMENTS IN SAMPLES FROM 17 PATIENTS WITH HYPERPARATHYROIDISM AND IN 4,900 MEASUREMENTS IN BONE FROM 17 CONTROL INDIVIDUALS. The mineral content is expressed in the thickness of aluminum foil that gives the same X-ray absorption as 100 μ of bone. In hyperparathyroidism the mean is 74 μ, with a standard deviation of 9.2. In the controls the mean is 84.6, with a standard deviation of 7.6.

	BONE DESTRUCTION BONE FORMATION	DECREASED MINERAL CONTENT OF BONE (Routine X-Ray)
NORMAL INDIVIDUAL	← →	—
COMPENSATED HYPERPARATHYROIDISM	← →	—
DECOMPENSATED HYPERPARATHYROIDISM	← →	+

FIG. 7. HYPOTHETICAL EXPLANATION OF THE ABSENCE OF ABNORMALITIES OF THE SKELETON IN HYPERPARATHYROIDISM.

seeking isotopes than that of old individuals (16). The crystal size of subperiosteal bone from the human rib was shown to be larger in old individuals than in young ones (17). Investigations have also been done on bone structures of various ages. The mineral content of young structures is lower than that of older structures (18). In the bone of the human fetus mineralization takes place very rapidly in the first five weeks of this process, but after this period the continued rise in mineral content is very slow (19). Comparable results were found for the calcification in callus formation in rabbits; 60 weeks after the beginning of mineralization the maximum had not yet been

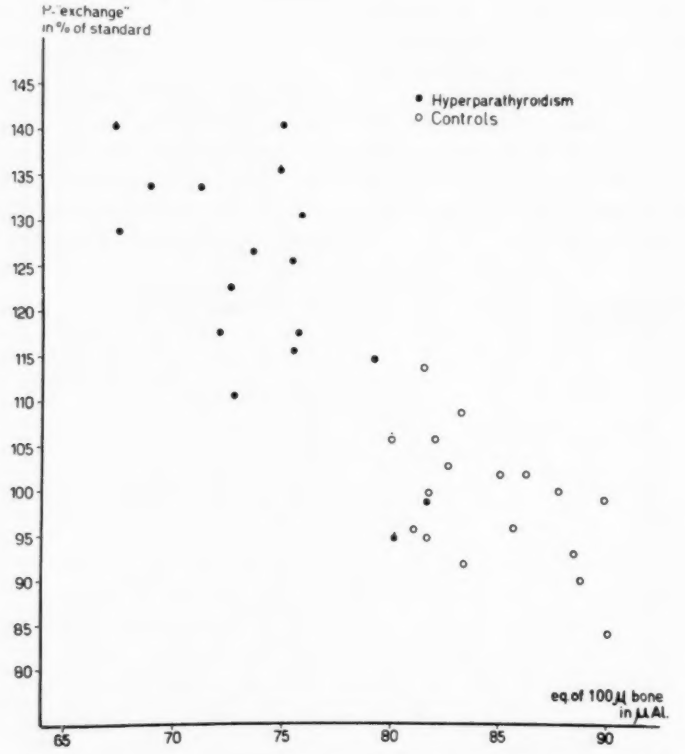

FIG. 8. NEGATIVE CORRELATION BETWEEN THE MINERAL CONTENT AND THE *IN VITRO* PHOSPHATE "EXCHANGE" IN BONE FROM 17 PATIENTS WITH HYPERPARATHYROIDISM AND 17 CONTROLS. The mineral content is expressed in the thickness of aluminum foil that gives the same X-ray absorption as 100 μ of bone. The phosphate "exchange" is expressed as a percentage of a standard. A 61 year old patient, with hyperparathyroidism and cystic lesions in the skull and in a vertebra, had a normal phosphate "exchange" (95 per cent) and a normal mineral content (80.1 μ Al) of bone from the iliac crest.

reached (20). These data are in agreement with the concept that young bone contains smaller bone crystals than old bone (9).

The results obtained with the phosphate "exchange" experiments with bone from individuals of different ages as described in this paper, indicate that the exchange in young individuals is higher than that in old ones. Since the average crystal size in young individuals is smaller than that in older ones, the crystal surface will be relatively larger, as will the space between the crystals, which is important for diffusion (9). The "exchange" possibilities for ions from the crystal are thus increased.

The investigations of the bone of patients with hyperparathyroidism indicate that the phosphate "exchange" is higher and the mineral content lower than in bone from the controls. Both phenomena can be explained by assuming that, on the average, the crystal size in hyperparathyroid patients is smaller than that in normals. Since there is no indication that the total amount of skeletal bone is increased in hyperparathyroidism (as far as is known only a decrease can be observed), the small crystal size can only be explained by a relative increase in low mineralized bone.

The most obvious explanation for these phenomena is the assumption that new bone formation is accelerated in hyperparathyroidism (Figure 7). This explanation is only valid if the characteristics of young bone, as they are known from healthy individuals, are the same in bone from hyperparathyroid patients. The increase in osteoplastic activity found in hyperparathyroidism (21) supports this hypothesis. Alternative explanations, e.g., generalized demineralization or a slowed rate of mineralization can not be excluded.

The results obtained in this investigation of human bone samples are in agreement with those obtained with qualitative microradiography of bone in experimental animals (7). The results of recent work with radioactive calcium isotopes (22), and with stable strontium (23), in humans with hyperparathyroidism, in which pools are calculated from disappearance curves, are also in agreement with our measurements in bone biopsy specimens.

It is striking that the 61 year old patient, with a mineral content equivalent to 80.1 μ aluminum and a phosphate "exchange" of 95 per cent in bone from the iliac crest (Figure 8), both normal values, showed roentgenologically and histologically an ostitis fibrosa cystica generalisata in several parts of the skeleton. This patient therefore had a local bone disease which could not be demonstrated in the iliac crest, and shows the reverse of what is shown by most of the other patients in whom no localized bone disease could be demonstrated but in whom there were abnormalities in the bone of the iliac crest.

As was to be expected, a reverse correlation is found between the mineral content of the bone and the readiness to exchange phosphate ions *in vitro*. The correlation coefficient for the 17 patients with hyperparathyroidism is -0.69 ($p < 0.001$), and for the hyperparathyroid group and the control group together, -0.62 ($p < 0.001$). In Figure 8 the relation between these two investigative methods is shown graphically.

SUMMARY

1. A phosphate "exchange" test *in vitro* with bone powder and a P^{32}-labeled phosphate solution was used on human bone powder prepared from biopsy samples from the iliac crest. The results of the phosphate "exchange" experiments are expressed as a percentage of a standard, which had an absolute exchange capacity of 26.8 per cent after 24 hours of incubation.

2. The results of the phosphate "exchange" test in a group of 80 human controls show an inverse correlation between the phosphate "exchange" of the bone and the age of the individual ($R = -0.69$, $p < 0.001$). An inverse correlation between phosphate "exchange" of the bone and the age of the individual was also shown in an experiment with rats.

3. The phosphate "exchange" *in vitro* is greater in bone from patients with hyperparathyroidism than in bone from controls. The relative "exchange" percentages are 123.2 ± 13.2 and 99 ± 6.9 per cent ($p < 0.001$).

4. Bone from five patients with hypercalcemia not caused by hyperparathyroidism had an average "exchange" percentage which agreed with that for the control group.

5. A technique of quantitative microradiographic determination of the mineral content of bone ob-

tained by biopsy from the iliac crest using aluminum foil as a reference standard, is described.

6. The bone of 17 patients with hyperparathyroidism had, on the average, a lower mineral content (100 μ bone equals 74 $\pm$ 4 μ aluminum) than that of 17 control individuals (100 μ bone equals 84.6 $\pm$ 3.3 μ aluminum; $p < 0.001$).

7. Five patients with hypercalcemia not due to hyperparathyroidism had, on the average, a normal mineral content of bone (100 μ equals 82 μ aluminum).

8. There is a significant correlation between the mineral content of the bone and the phosphate "exchange" percentage ($R = -0.62$, $p < 0.001$).

9. Both the high phosphate "exchange" of the bone from patients with hyperparathyroidism and its low mineral content can be explained by assuming an increased bone production in compensation for the increased osteoblastic bone destruction.

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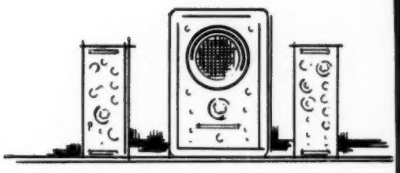

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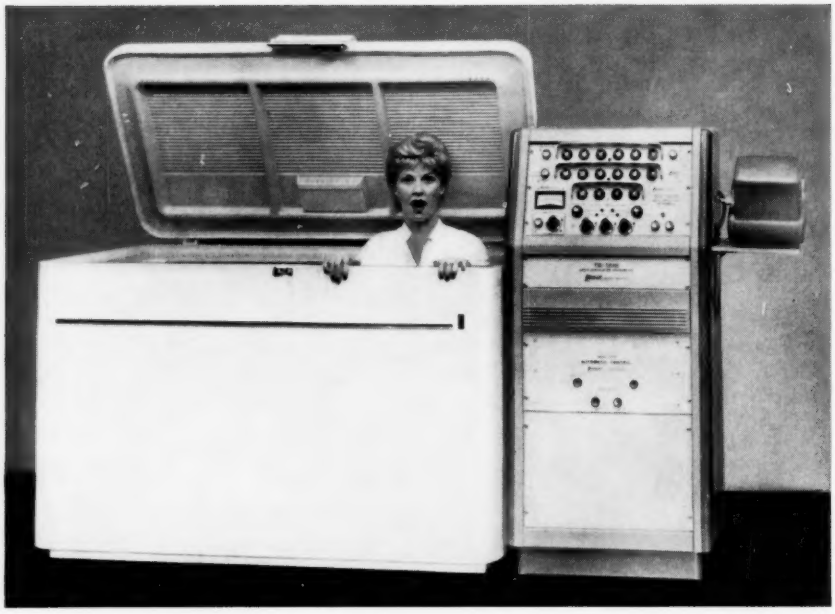

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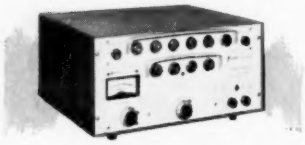

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